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Sequential Sporangiospore- Yeast Transformation Hypothesis

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A. PLEOMORPHISM

The first source of isolation of *Dimorphomyces* strains was the soursop microenvironment (Omoifo, 1996a). Multiple forms of the strains were observed during their cultivation but neither zygospores nor chlamydospores have been detected. Soursop extract induced several morphologies from sporangiospores of *D. pleomorphis* strain C13, IMI W5132B including terminal budding yeast cells, multipolar budding yeast–like cells, pseudomycelia with multilaterally borne blastospores and holoblastic conidia. All of these

remained as sediment, or in suspension, but fertile coenocytic sporophores shot into the atmosphere after one month of growth. Micrographs of such morphologies were shown in fig. 1-10. Since the different morphologies were induced from sporangiospores of a single type of pure culture, it meant that the different forms were organically connected, and that the organism existed in a multiplicity of asexual reproductive states. It also meant that the microorganism was pleomorphic. We have designed complete artificial media for the propagation of some of the imperfect morphological states (Omoifo, 1996b, *in press*).

D. pleomorphis strain C13, IMI W5132B was the first to be used in extensive studies (Omoifo, 1996a,b). This was followed by *D. diastaticus* strain C12, IMI W5132A (Omoifo, 1997; *in press*) and, subsequently *D. ubiazae* strain C16, IMI W5132C (*in press*). These strains were also found to exist in multiple asexual states but with less anamorphic possibilities than that of strain C13, IMI W5132B. An inherent element in the life pattern of these strains was their ability to transform from one morphological form to the other, a fit apparently higher for strain C13, IMI W5132B considering the higher number of anamorphs and lack of additional stimulatory factors for such conversions. For instance, strain C12, IMI W5132A required uracil to transform to the terminal budding yeast morphology (Omoifo, 1997) while strain C16, IMI W5132C had absolute requirement for elevated temperature for conidiogeneously produced terminal budding yeast morphology (*in press*). Furthermore, strain C13, IMI W5132B had higher number of asexual states, in simple and complex media, in comparison with strain C12, IMI W5132A. It was reasonable to conclude, therefore, that of the three strains, strain C13, IMI W5132B had a higher transformability potential.

In aerobic growth environment, the strains exhibited mucoraceous affinities with the production of multispored sporangia as the main feature. In chemically defined broth, the sporangiospores transformed to terminal budding yeast cells, a distinct morphology that literature has not revealed to be associated with *Mucor* species but rather specific to the class, Ascomycetes, although it is also associated with the Basidiomycetes and Fungi Imperfecti. However, strain C13, IMI W5132B transformed to multipolar budding yeast-like cells, a morphology that is possible with many mucorales in modulated environments.

The multipolar budding yeast-like morphology of strain C13, IMI W5132B occurred naturally in soursop extract (Fig. 7-9, 11), the port of first isolation. It also occurred in simple medium, but with complex nitrogen source. The high-sugar derived pseudomycelial morphology of strain C13, IMI W5132B (*in press*), also a feature of the natural environment (Omoifo, 1996a, 7, 11), might be similar to that produced by *Candida* species (Kreger Van Rij, 1984). But characteristically, the blastospore of strain C13, IMI W5132B, on seceding was non-budding. This morphology contrasts with that of *Saccharomyces rouxii,* which is also sugar tolerant (Koh 1975a, b; Hawker, 1966.) but exhibit terminal budding yeast morphology.

Interest in the intriguing characteristics of these organisms must also extend to differences in colonial features. In agar medium the abundant mycelia growth of strain C12, IMI W5132A was white in color, whereas strain C13, IMI W5132B, with less abundant filamentation had olive-orange color, which could be bleached under white light after 15 days of growth (Omoifo, 1996a). On the other hand strain C16, IMI W5132C remained olive orange throughout the growth period (*in press*). Initial aerial mycelia of strain C13, IMI W5132B was upright but became lodged or bow-shaped after 3 days in contrast to the vigorous growth of strain C12, IMI W5132A and strain C16, IMI W5132C both of which remained upright through one month. Of the 3 strains, strain C13, IMI W5132B was the slower growing. In one study, it was shown that the ability to colonize agar media by strain C12, IMI W5132A and strain C13, IMI W5132B differed greatly. Whereas strain C12, IMI W5132A had 75% and 64% colonization, respectively in organic nitrogen and inorganic nitrogen media buffered at pH 4.5, those for strain C13, IMI W5132B were 55 and 47.5% respectively for similar media. The most remarkable distinction occurred in synthetic broth in which no two of the strains had the same procedure for induction of terminal budding yeast morphology (Omoifo, 1996a,b 1997; *in press*); strain C12, IMI W5132A requirement: maltose-substrated uracil-incorporated multiionic broth, temp 20^{o}C, pH 3.5; strain C13, IMI W5132B requirement: glucose–substrated multiionic broth, 20^{o}C, pH 4.5; strain C16, IMI W5132C requirement: glucose-substrated multiionic broth, temp 37^{o}C, pH 4.5. Considering the differences in colonial characteristics, procedure for terminal budding yeast morphology induction, anamorphic possibilities, still, coupled with biochemical differences (Omoifo, 1996a) one was led to the conclusion that these strains were distinctly separate microorganisms. However, the similarities in hyphal structures and septation, the inability to detect columella in the multispored sporangia borne at the apex of coenocytic sporangiophore, probably suggest the same generic origin.

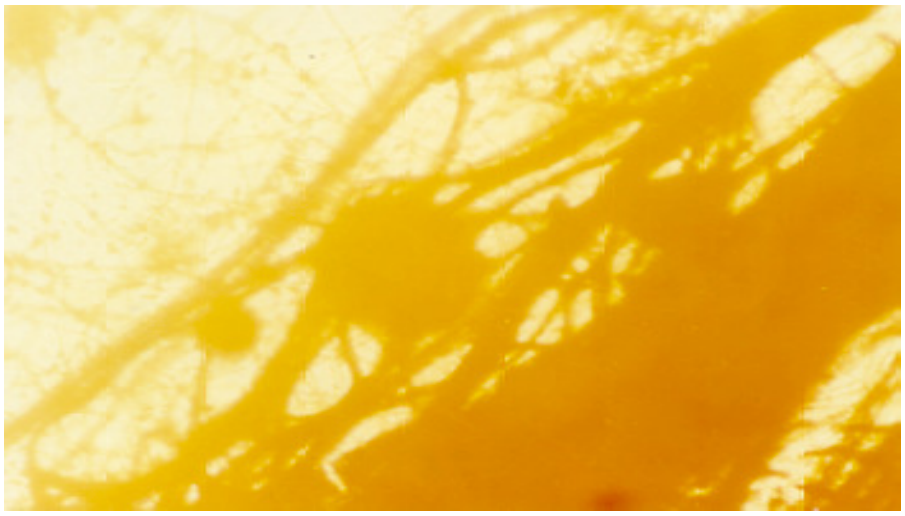


Fig. 1: Mycelia and sporangium of strain C13, IMI W5132B induced in soursop extract after 1 month of growth at 28±1°C ambient. Mag. x 100

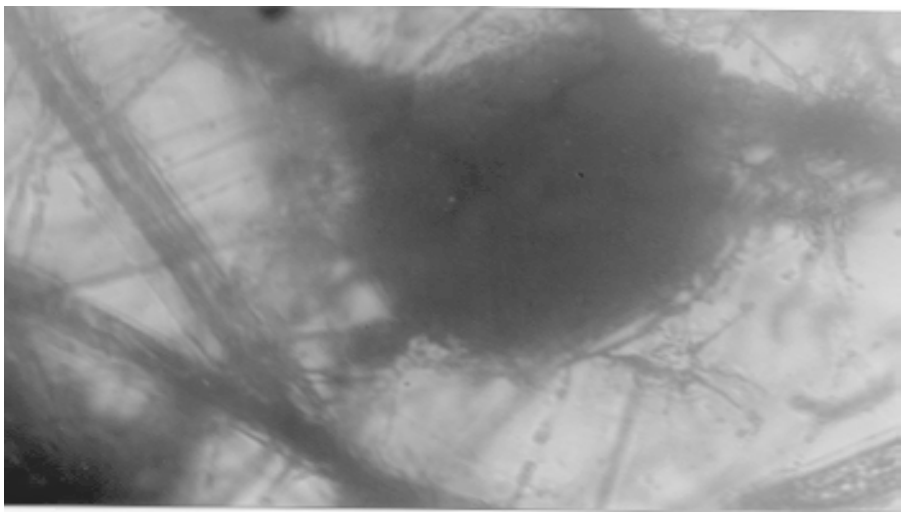


Fig. 2: A higher magnification of sporangium of strain C13, IMI 5132B induced in soursop extract after 1 month of growth at 28±1°C ambient. Mag. x 400

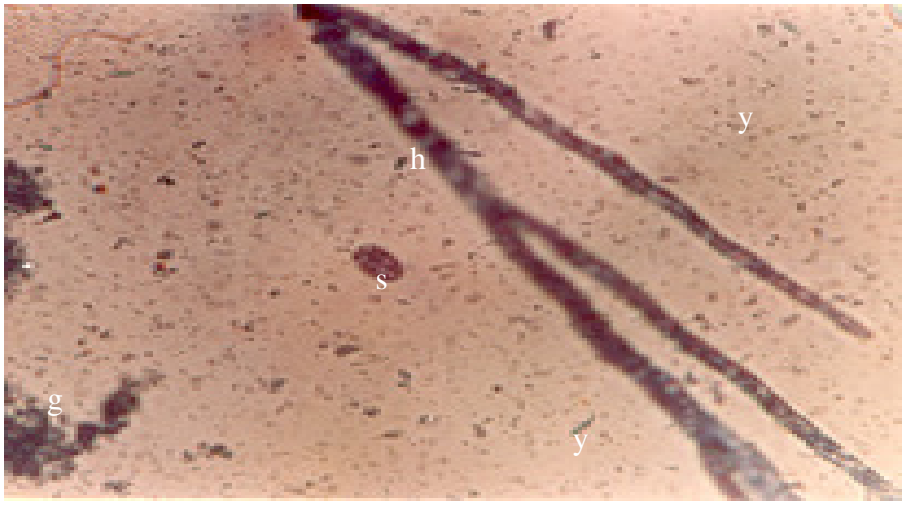


Fig. 3: Sporangiospore (s), branched hypha (h), yeast cells (y), and clumps of granular particles (g) of strain C13, IMI 5132B induced in soursop extract after 1 month of growth at 28±1°C, ambient. mag. x400

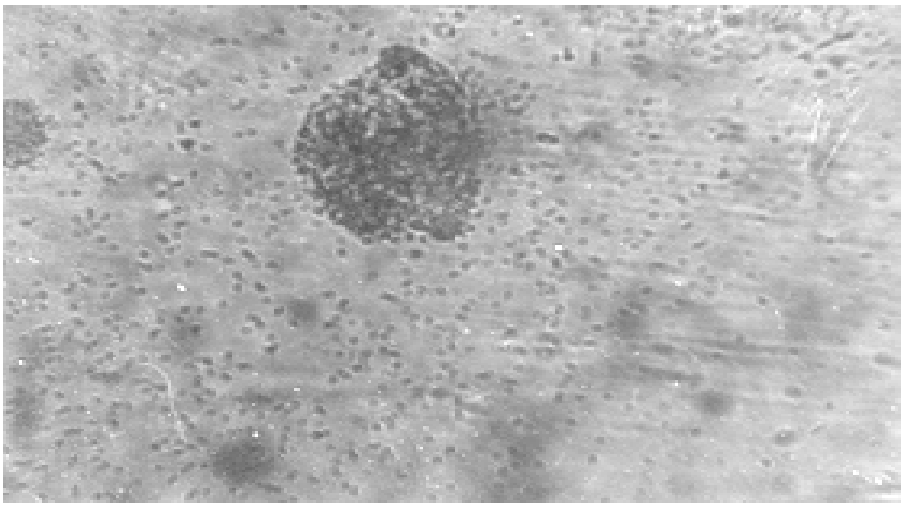


Fig. 4: Cytoplasmic granular particles of strain C13, IMI W5132B, from which the enclosing sporangiospore wall had lysed, in soursop extract after 3 days of growth at 28 ± 1°C, mag. x 400.

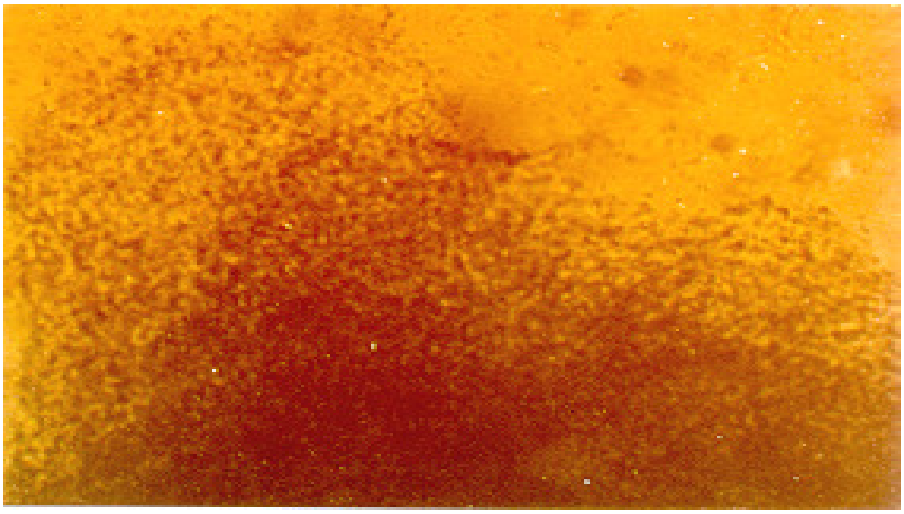


Fig. 5: Spreading granular particles of strain C13, IMI W5132B, induced in soursop extract after 3 days of growth at 28 ± 1°C, mag. x 400.

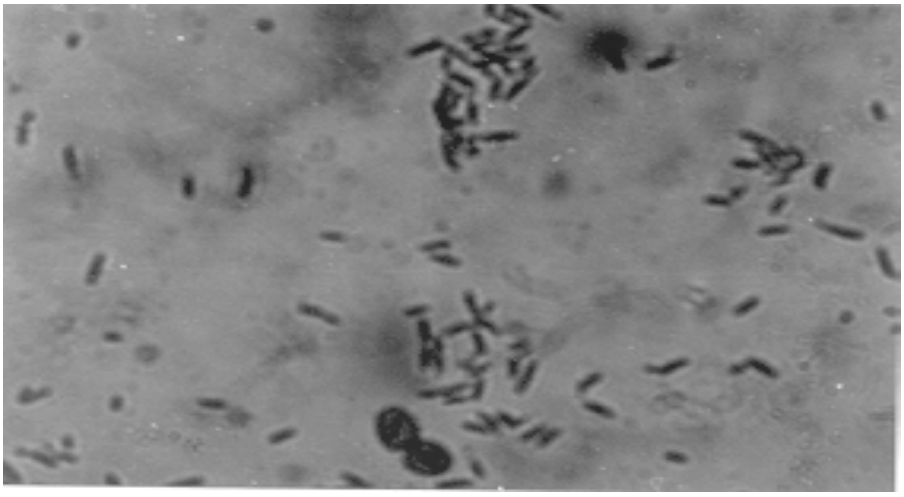
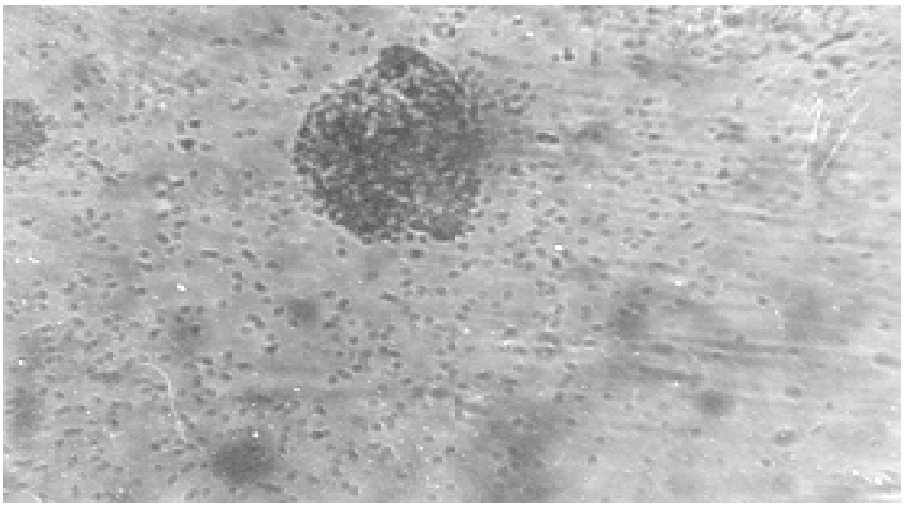


Fig. 6: Terminal budding yeast cells and holoblastic conidia of strain C13, IMI W5132B, induced in soursop extract after 3 days of growth at 28 ± 1°C, mag. x 400.



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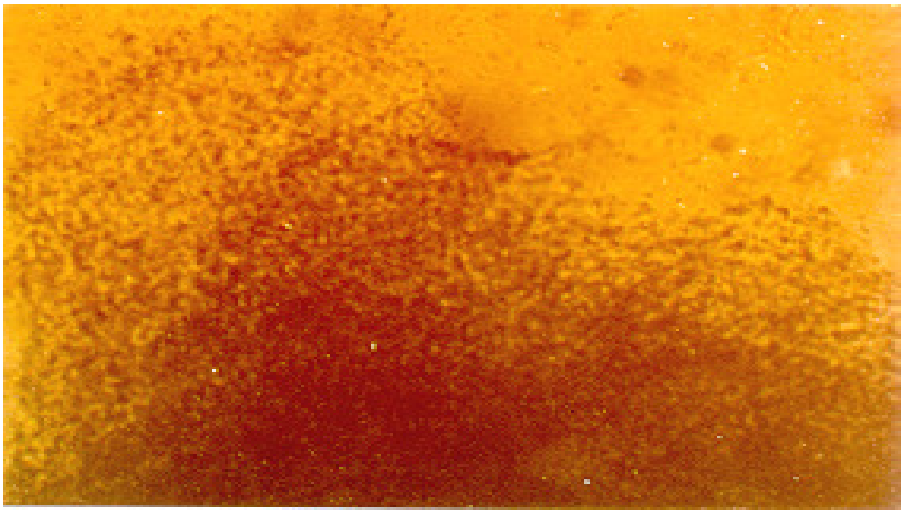

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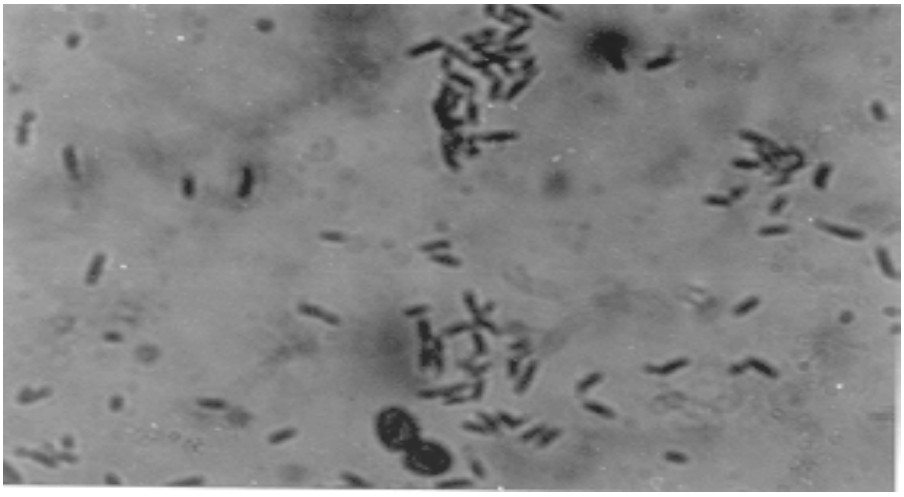

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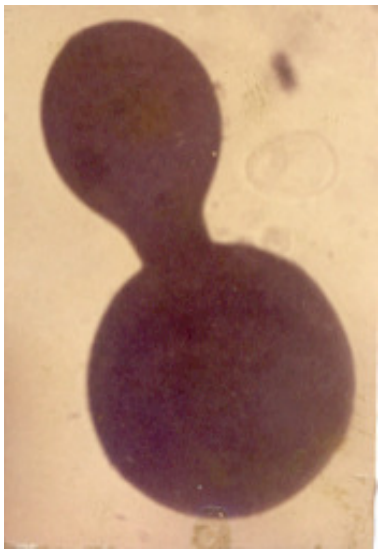

Fig. 10: Yeast cell of strain C13, IMI W5132B, induced in soursop extract after 1 month of growth at 28 ± 1°C, mag. x 400. Note: the yeast cell was unusually large; compare fig. 3 and 6 of similar magnification.

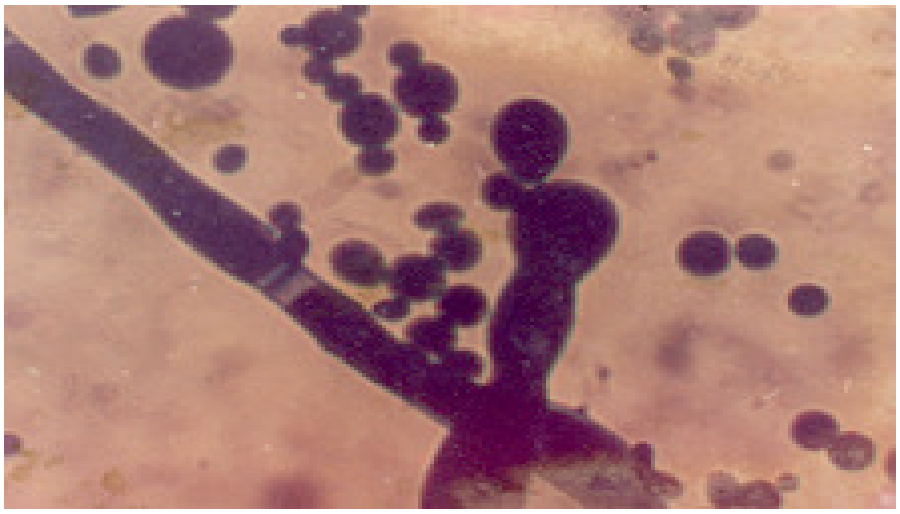

Fig. 11: Yeastlike cells and pseudomycelia of strain C13, IMI W5132B, induced in soursop extract after 1 month of growth at 28±1°C, mag.400.

In 1996, the International Mycological Institute Kew, Surrey identified the strains as *Mucor circinelloides* Tieghe. Despite this, some publications have been made under the tentative form-genus *Dimorphomyces* (Omoifo, 1996a, b; 1997). The cultures sent for identification were also said to be a mixture of several fungi, yeasts and bacteria. This was perhaps true. Perhaps also true was the fact that because of the pleomorphic nature of each of the strains, the representative anamorphs were recorded as distinct microorganisms. Of particular note were the cytoplasmic units which on growth sphere cell wall lyses were released as granular units, and protoplasts on the one hand, and active units and emerging yeasts on the other, observable in the transformation process of each of the strains (Omoifo, 1996a,b 1997; *in press*) that could be similar to bacterial cells. Also, terminal budding yeast cells, a characteristic of the higher classes of fungi were conspicuously expressed in each induction procedure. Further differences between the mucoraceous state of the dimorphic strains and *M. circinelloides* Tieghe were shown in table 1.

Worthy of note was the fact that sporangiospores of strain C12, IMI W5132A grew as pseudomycelia, which produced endospores in PDA slide cultures whereas pseudomycelia of strain C13, IMI W5132B produced blastospores in similar medium, and the latter strain was the only one known to bud multipolarly. On the other hand, strain C16, IMI W5132C in addition to holoblastic conidia, thallic conidia, which the other strains similarly produce, also produce enterothallic conidia (*in press*).

Teleomorph, a key factor in the taxonomy of the perfect fungi, has not been found in these strains. It was suggested that the characteristics of the dimorphic strains hereby discussed place them outside the characteristics of the order Mucorales, as outlined by Hesseltine and Ellis (1973) and Alexepoulos and Mims (1979). Since classification would be more appropriately based on natural affinities, perhaps the multiplicity of anamorphs in the life pattern of each of these dimorphic strains would consign them to the Fungi Imperfecti, which accommodates pleomorphic microorganisms with yet undetected teleomorphs (Hennebert, 1971)

B. SEQUENTIAL SPORANGIOSPORE - YEAST TRANSFORMATION HYPOTHESIS

i. Introduction

In the study of Omoifo (1996b), several factors including pH, temp and media-type were integrated into a unified sporangiospore-growth environment, and in an attempt to determine combined and individual effects on the proliferation of strain C13, IMI W5132B subjected the growth response to statistical analysis. Four different morphologies including spherical cells, granular units, yeast cells and yeast-like cells emerged depending on the interactive combination of the growth factors. Granular units were observed to be important for the successful conversion to the yeast phase, but the yeast-like phase could be terminal depending primarily on nitrogen source and incubation temp. This was akin to findings in the earlier literature on nutrient and temperature dependent dimorphism (Romano, 1966).

Apart from mould-yeast-like interconversion, other patterns recognized in literature include mould-yeast interconversion, mould-pseudomycelia interconversion (Romano, 1966). Hypotheses have been proposed as theoretical working tools for further studies on fungal dimorphism. For instance, Nickerson and Edwards (1949) proposed that there was selective inhibition of cell division without simultaneous effect on growth and that thermal inactivation of enzymes might play a leading role in the conversion of mould to yeast-like cells. While Pine and Peacock (1958) premised their model on the involvement of metabolic route in the conversion process, Kanetsuna and coworkers (1972) hinged yeast-like development on the molecular architecture of the cell wall whereby less of rigidity around β − glucan component create soft spot for formation of daughter bud. On the other hand, Stewart and Rogers (1978) like Bartnicki-Garcia (1973) premised yeast-like development on the differential rate of cell wall growth.

In this section we have identified a population with transient morphology (TM) in the conversion process, from sporangiospores to terminal budding yeast cells. The occurrence of granular cytoplasm is not new in literature, as they are observable in the micrographs shown in the experiments of Bartnicki-Garcia and Nickerson a, b, c; Clark- Walker, 1973; Friedenthal *et al.*, 1974; Haidle and Storck, 1966; Hall and Kolankaya, 1974; Jones and Bu'Lock, 1977; Larsen and Sypherd, 1974; Paveto *et al.,* 1975; Zorzopulos *et al.,* 1973.

We have employed concepts from other disciplines to explain this phenomenon, which subsequently led to formulation of a unified transformation hypothesis on sporangiospore- yeast conversion of dimorphic fungi.

Table 1: Some differences between M. circinelloides Tiegh and the mucoraneous state of the dimorphic strains.

Character	*Mucor circinelloides* 252	Mucoraceous state of the dimorphic strains
Colony	Grey at first, then brown	W5132A: white W5132B and W5132C: olive orange
Matyre sporangia	Brown	W5132A: gray W5132B and W5132C: olive orange
Sporangial wall	Warty	Smooth
Sporophores	Upright with circinate Lateral branches	Upright, but strain W5132B bow After 3 days
Columellum	Globose to subglobose	Not detected
Sporangiophores	Subglobose	In GYPA W5132A: globose To ellipsoidal; W5132B; ellipsoidal; W5132C; globose to ellipsoidal
Sporangiophore mass	Black-and-gray	W5132A; white-and-gray W5132B: orange W5132C: orange
Mycelium: bottom top	Dense Sparse	Dense W5132A: Dense W5132B: less dense W5132C: dense
Mycelium: height	2-10mm	W5132A: > 10mm W5132B: 4 – 10mm W5132C: > 10mm
Hypha	Non-septate	Septate
Chlamidospores	Present	Not detected
Place of isolation	Soil, dung and Rotten potato	Spontaneously fermented Soursoup extract
Fermentation	Dextrose, fructose, maltose	W5132A: Dextrose, galactose, lactose Maltose, sucrose, soluble starch, Raffinose; W5132B: Dextrose, galactose, lactose, Maltose, sucrose, soluble starch, Raffinose; W5132C: not tested

*Characteristics as published by Fassatiova (1986)

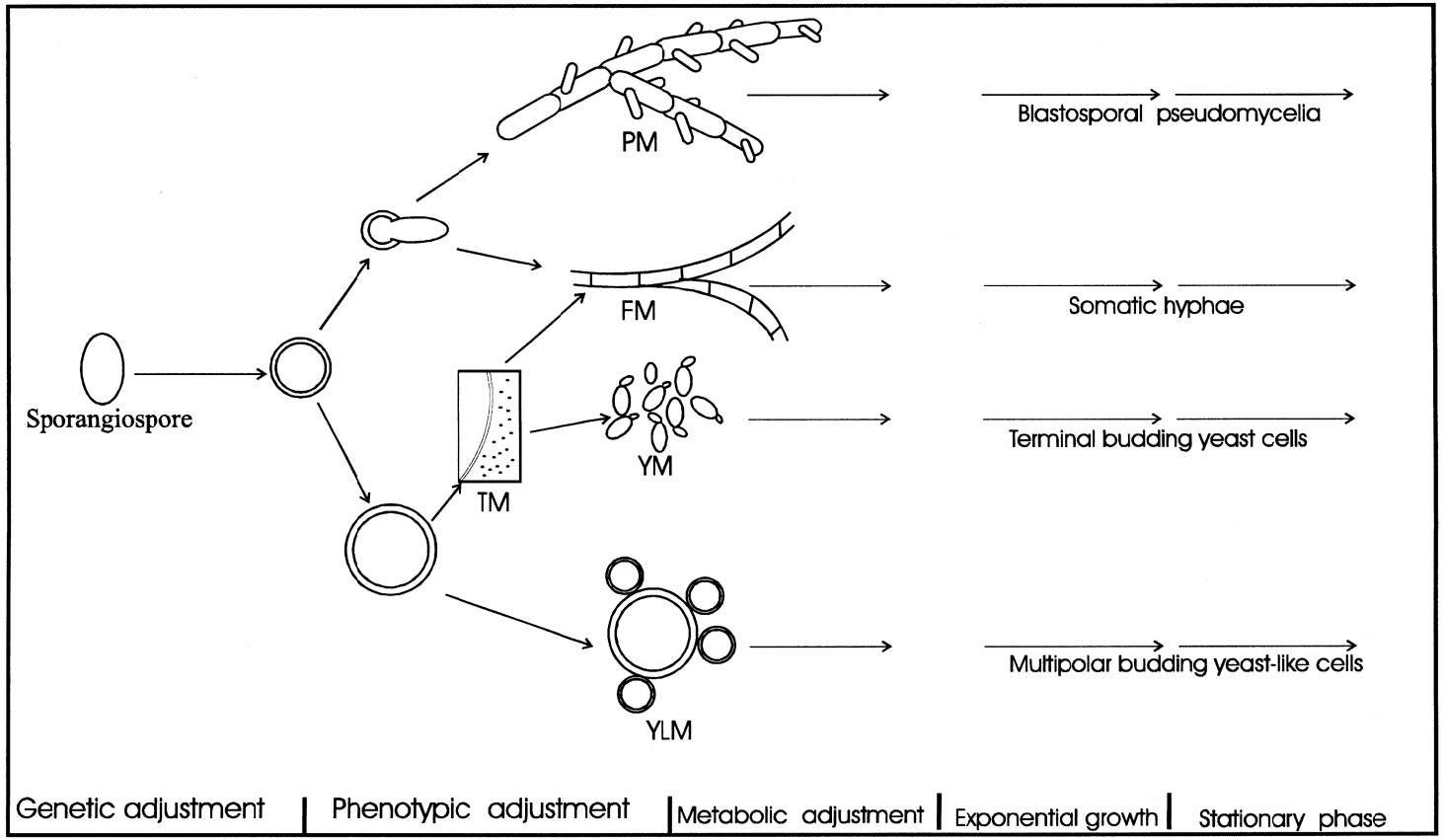

Fig. 12: Scheme illustrating morphological and growth phases of induced forms from sporangiospores of strain C13, W5132B. The presence of a population of TM (transient morphology) indicated the direction of development for yeast morphology (YM) expression. FM = FIlamentous morphology, PM = pseudomycelia morphology, yeast - like morphology.

ii. The Hypothesis: Sporangiospore-yeast Transformation

Factors such as ionic strength, semipermeable membrane, transmembrane-pH- gradient which influence entry of ions and, or osmotic regulators into the cytoplasm enable the sporangiospore to undergo isotropic growth; the flux of ions very likely effect changes in the membrane potential as well as electrostatic potential that determine the capability of the nuclear materials to initiate biosynthetic and replicate activities, hence forming individual units within the isotropically growing non-dividing cell with thick wall but on lyses, the granular units (GUs) become exposed to the growth medium. A graphic illustration of the growth process and lyses was shown in Omoifo (1996b). The generation of GUs by the dimorphic strains has been documented in several studies (Omoifo, 1996a, b; 1997; *in press*). Careful examination of results in these studies emphasized the importance of sequential development in buffered multiionic broth and has led to the formulation of a unifying hypothesis, which we called sequential sporangiospore-yeast transformation (SSYT) hypothesis.

The transformation of sporangiospores of **Dimorphomyces** species in defined buffered multiionic broth with glucose as the only preformed organic source occurs in sequence. At any given moment, in response to electromotive transport mechanisms, isotropic growth (IG) of spores occurs during which synchronous DNA replication supervene thereafter differentiating into individual cytoplasmic units. Thus, the growth sphere appears granular. By modulation of the transport control mechanisms, the cell walls lysed hence giving rise to a population with TM in the form of GUs. The relative size and content of individual units of this GU population at low metabolic status gradually become protoplasmic. This subsequently assumed the yeast morphology, which thereafter become terminal budding. Transfer of the granular units or protoplasts unto solid media under aerobic conditions results in filamentous morphology (FM). But modulation of isotropic growth by elevated temperature and complex nitrogen source will cause the non-lysing of the cell wall and rather give rise to multipolar budding yeast-like morphology (YLM) whereas imposition of osmotic stress by hexose induces pseudomycelia morphology (PM). The formation of 'stable morphology' i.e. YM, YLM, PM, or FM is therefore followed by the normal growth pattern. The hypothesis is diagrammed in Fig. 12.

During IG, the generation and evolution of individual units was assumed to stem from synchronous DNA replication triggered by modulation of electrostatic potential of ionic species in the nuclear atmosphere. Members of the population of TM thus evolved and released after cell wall lyses, increase their relative sizes, and therefore actively growing, presumably resulting from proton-substrate symport, and hence metabolizing in a fermentative manner, until assumed a permanent proliferate (mitotic) or budding morphology (Omoifo, 1996b, 1997). The terminal phenotypic expression of YM or FM was thus dependent on the growth conditions of GUs, whereas in the case of YLM and PM, GUs never was released from the growth spheres.

iii. Support for the hypothesis: Identification of a transient phase

Sporangiospores (3.5µ) of strain C13, IMI W5123B (Omoifo, 1996a) like those of *M. rouxii* (Bartnicki-Garcia *et al.,* 1968) and conidiospores of *Aspergillus niger* (Anderson and Smith; 1972; Yanagita, 1957) undergo exogenous swelling resulting in spherical growth in nutrient medium, including strictly defined broth with glucose as the only preformed organic compound. Such volume changes, dependent on medium osmotic pressure (Alemohammad and Knowles, 1974; Knowles, 1971; Knowles and Smith, 1971) resulted from facilitated diffusion and active transport mechanisms, which allowed solute influx through the plasma membrane (Dawes, 1986; Jain, 1972; Kaback, 1972; Rose, 1976). This osmotically induced volume changes, metabolically regulated (Alemohemmad and Knowles, 1974) might also be termed isotropic growth (Anderson 1978), which results in a growth sphere (Bartnicki-Garcia and Lippman, 1977). This was the morphology derived from the early growth phase of strain C13, IMI W5132B in buffered multiionic broth, pH 2.5, 3.0, 3.5, 4.5, and 5.5, temp 15^{o}, 20^{o}, or 37^{o}C. In that study, IG cell (growth sphere) was termed 'enlarged globose cell' (Omoifo, 1996a,b). Such cell was clearly larger at 37^{o}C and was in agreement with the finding of Anderson and Smith (1972) who noted faster growth rate and larger sizes for spores of *A. niger* incubated at 38^{o}C, in contrast to the size at the lower temperatures.

Our study showed that during IG, the buffered growth medium pH decreased to a minimum, at 60h after incubation (Omoifo, 1996b). The steady decrease was interpreted as an indication of electrogenic proton extrusion from intracellular medium, deemed to have occurred in direction and space as in mitochondrion (from intracellular medium to the intermembrane space; Mitchell, 1967). Following imbibitions, it was likely that redox reactions, in the presence of pH differential and internal buffer capacity

and, against external buffered medium set up a proton concentration gradient across the plasma membrane of growth spheres with consequent electron influx (Dawes, 1986; Harold, 1977; Rose, 1976). This then subjected the growth spheres to classical membrane phenomenology whereby receptor-ligand interaction created primary ionic pathways, ionic conductivity, influenced by intrinsic protein resistance which showed inverse relationship with membrane permeability (Eaton, 1985). In such biological system with differential external and internal ionic concentrations, a relationship was therefore envisioned through Goldman-Hodgkin Katz physicochemical measures.

Membrane potential (E_m) was an event a number of steps removed from the initial causative interaction (Eaton, 1985). Cone (1974) linked E_m generation with mitotic activities, metabolically modulated through a system of feedback circuits and switching mechanisms, and evidence was provided to show that depolarization of the membrane resulted in initiation of DNA synthesis and mitotic activity leading to cell proliferation. However, the controlling role of Na^+ ion in mitogenic regulation was emphasized (Cone, 1974,1980; De Laat and Saag, 1982; Sparks *et al.* 1982). An addendum to his original theory re-emphasized the strong involvement of E_m in the physiological and morphogenetic regulation of cells (Cone, 1985).

In our study, the decreasing pH of the growth medium presumably resulting from proton extrusion from growth spheres of strain C13, IMI W5132B was seen as causing membrane depolarization and, hence triggered DNA biosynthesis. The profoundly numerous GUs could have resulted from DNA replication thus giving rise to multinucleate of the growth sphere. Bartnicki- Garcia and Nickerson (1962b) used HCl. Giesma staining to show that spherically grown cells of *M. rouxii* were multinucleated.

If we realized that in multinucleated *Physarium polycephalum,* the nuclei divided synchronously (Chin and Bernstein, 1968; Haword, 1932) in the absence of cell division (Chin and Bernstein, 1968), and that mitotic synchrony was a feature of lowered membrane potential (E_m) induction when some substances pervade the cytoplasm to establish the mitotic environment around each nucleus thereby keeping them in step through division (Cone, 1971), steps therefore occurred in which premitotic ATP build– up was utilized in driving the mitotic process (Chin and Bernstein, 1967,1968). These pervading substances, as Maurel and Douzou (1978) explained, polyelectrolytically modulate the electrostatic potential in such microsystems where local changes in electrostatic potential were considered in terms of perturbations of the interdependent pH – ionic strength relationship; they would alter the mitotic environment for catalytic enzyme activity (Maurel and Douzou, 1978). We thus saw the value in assuming that at the fundamental level, DNA replication was given a magnitude by the pH-ionic strength effect, as did Ca^{2+} ion in modulating protein kinase C activity which on the one hand, caused increase in cytoplasmic pH, sequel to onset of DNA synthesis (Rozengurt, 1986) and on the other, effected phosphorylation at the genome promotion site, activated by the cAMP response element binding (CREB) protein (Lalli and Sassone-Corsi, 199; Voet and Voet, 1995). In the event of these activities occurring during the cultivation strain C13, IMI W5132B in the buffered multiionic broth (Omoifo, 1996b), then these could be sequentially followed by transcription and translation that probably resulted in cytoplasmic granulation. Cytoplasmic granulation has been observed in many microorganisms (Table 2).

The buffered multiionic broth used in strain C13 W5132B cultivation contained polycations and polyanions (Omoifo, 1996b), which in solution might qualify for Maurel and Douzou's (1978) permanent and mobile charges. Cone (1971) explained that Na^+ ion (a constituent of our buffered synthetic broth) conductivity and Na^+ ion flow rate determine E_m level; furthermore, Na^+ efflux was simultaneously accompanied with K^+ influx (active) and Cl^- efflux (passive). It was also noted that cellular membranes could have several ionic permeability systems (Eaton, 1985). Such ions carry mobile charges that interact with structural components of the cell wall. Goldman rectification recognizes that changes in permeability are brought about by similar changes in E_m and, possibly, membrane current. Thus, mobile ion changes within the membrane create the microsystems: enzyme- polyelectrolyte (Maurel and Douzou 1978). Polyelectrolytes help to determine the ionic strength.

Reference has been made to the pH- ionic strength interdependence. Maurel and Douzou (1978) reported that at sufficiently high ionic strength when electrostatic potential in the polyelectrolytic phase became lowered through the mobile charge distribution, lyses of ß 1-4 glycosidic bonds in the structural polysaccharide materials of cell wall of *Micrococcus luteus* occurred at acidic pH levels. Glycosidic bonds have been shown to be cell wall structural linkages of polysaccharide materials of dimorphic species. Kanetsuma and coworkers (1972) found α 1-4 glycosidic linkages in walls of isotropic cells of *Paracoccidioides brasiliensis,* but fungal walls contained β 1-3 glycosidic bonds with β 1-6 linkages at the branch points. If optimum conditions of pH and ionic strength were met, polycationic lysozymes would

Table 2. Granulation of cytoplasm has been observed in many microorganisms.

Organism	Medium	Granular Cytoplasm YLM	Gs	PM	Lyses	Reference
M. rouxii	complex (YPG)	+		+	-	Bartnicki-Garcia and Nickerson, 1962a
M. rouxii	complex (defined thiamine + nicotinic acid)	+		+	-	Bartnicki-Garcia and Nickerson, 1962
M. rouxii	complex (YPG)	+		+	-	Bartnicki-Garcia and Nickerson, 1962c
M. rouxii	complex (defined + 0.2% vit. free casamino acid)	+		+	-	Haidle and Storck, 1966
M. genevensis	complex	+		+	-	Clark-Walker, 1973
M. rouxii	complex (defined thiamine + nicotinic acid)	+		+	-	Zorzopulos *et al.*, 1973
M. rouxii	complex (YPG)	+		+	-	Friedenthal et al., 1974
Mycotypa microspera	complex (YPG)	+		+	±	Hall and Kolankaya, 1974
M. racemosus	Complex (YPG+ defined+0.2% casamino acids+ vitamins)	+		+	-	Larsen and Sypherd, 1974
M. rouxii	complex (defined + 0.2% casamino-acids)	+		+	-	Paveto *et al.*, 1975
M. hiemalis	complex (PDA. 2% malt extract agar	+		+	-	Jones and Bu'liock, 1977
D. pleomorphis strain C13, W5132B	complex (defined + peptone	+		-	±	Omoifo, 1996b
D. pleomorphis strain C13, W5132B	strictly defined	+		+	+	Omoifo, 1996b
D. diastaticus strain C12, W5132A	strictly defined		+		+	Omoifo, 1997

+, - denote positive and negative presence; at 20°C lyses of cell walls occurred while multipolar budding was observed at 37°C

likely cleave the glycosidic linkages in the polysaccharide network of cell wall of IG cells. The time rated study of sporangiospores of strain C13, IMI W5132B in buffered multiionic broth with extrinsically decreasing bulk medium pH with necessary accompaniment of a backward electron traverse, tend to suggest that intracellular conditions were optimum for lysozyme activity. When lyses occur the structural materials of the cell wall disintegrate into the growth medium and form part of the basal composition available for evolving phenotypic expression from a population of fine and coarse GUs (Fig. 13a,b, c).

Another structural shift occurred in the growth process of strain C13, I MI W5132B after cell wall lyses of growth spheres and this was accompanied by rising medium pH. Following our theory on transmembrane- pH- gradient, it was thought that proton uptake by the GUs thus commenced. Proton influx into organized system, an active process, was coupled with substrate uptake (Dawes, 1986; Jain, 1972; Rose, 1976; West and Mitchell, 1972,1973). Studies of Reber *et al.* (1977) showed that proton uptake by *Klebsiella aerogenes* was enhanced 1.5 times in the presence of scyllo-inositol. Accelerated rate of proton-amino acid -or – carbohydrate uptake has been demonstrated for mutant strains of *Saccharomyces cerevisiae, S. carlsbergensis* and *S. fragilis* (Seaston *et al.,* 1973). This coupled transport system occurred with specific ratios in microorganisms like *Escherichia coli,* 1:1 (West and Mitchell, 1972, 1973) *Metshnikowia reukaufii,* 1:1(Alderman and Hofer, 1981) and *Neurospora crassa,* 0.8.1.4 (Slayman and Slayman, 1974).

The GUs observed during the growth of strain C13, I MI W5132B constituted only a transient phase, for each increased in size apparently becoming protoplasmic and subsequently assuming the YM, initially seen in outline, but increased multidimensional until optimum size was achieved. Each cell became terminal budding. This was one of the morphologies expressed in the natural environment of *Dimorphomyces*; others include yeast-like cells, arthrospores, pseudohyphae, pseudomycelia that bear blastospores and vegetative hyphae (Omoifo, 1996a; Fig. 1-11). Figure 14 showed the sequential development of some of the morphologies that have been obtained in standard media.

iv. Significance of the SSYT hypothesis to the understanding of fungal morphogenesis

Concepts derived from this hypothesis call for greater consideration of principles evolved in other fields such as electrophysiology, genetic expression and intracellular communication and hence refocus on problem areas in fungal dimorphism. Some examples were provided.

Wertman and Paznokas (1981) observed no change in volume when sporangiospores of *M. racemosus* were incubated in minimal medium except with substrate added. The pH-time relationship during the cultivation of sporangiospores of strain C13, IMI W5132B was biphasic (Omoifo, 1996b). The SSYT hypothesis suggested that in hypertonic solution of high ionic strength, substrate was not necessary for isotropic growth, phase I, but its uptake commenced after cell wall lyses, phase II.

The identification of TM (Omoifo, 1996b; Fig. 3, 4, 5, 12, 13a-c) was central to the SSYT hypothesis. It was possible to monitor the nature and changing sizes of individuals of this population by facilities obviously not available to me.

It did not appear that the TM has been observed in other dimorphic fungi. We proposed that the evolving yeast cells grew by a mechanism, which occurred within the protoplast that linked proton-substrate symport with glycolysis and other biochemical processes that eventually gave rise to terminal budding yeast morphology. Fermentative carbon utilization by yeast-like cells has been demonstrated (Inderlied and Sypherd, 1978). However, cells with YLM were not glycolytically as efficient as those with YM (Bartnicki-Garcia, 1963). In another study, it was shown that two of the dimorphic strains were primed for wine production in the yeast morphology using soursop (*A. muricata* L.) extract as base. Although sensory evaluation was not conducted, chromatographic analysis showed that some of the major characteristics desirable in a good wine were inherent in the fermentation products.

Although strain C13, IMI W5132B occurred with several morphologies, YM being the main one, one study (*in press*) showed that buffering at the specific pH level was of absolute necessity for the development of YM in the synthetic broth. When non-buffered, such glucose-substrate multiionic broth induced all the aforementioned anamorphic states including pseudohyphae, which projected into the medium, septate vegetative hyphae on medium surface and aerial coenocytic sporangiophore with apically borne mulitspored sporangia. Carbon substrate not directly utilized in the glycolytic pathway also induced several anamorphic states. The role of buffering on the one hand, and carbon type, on the other, in yeast development need further investigation.

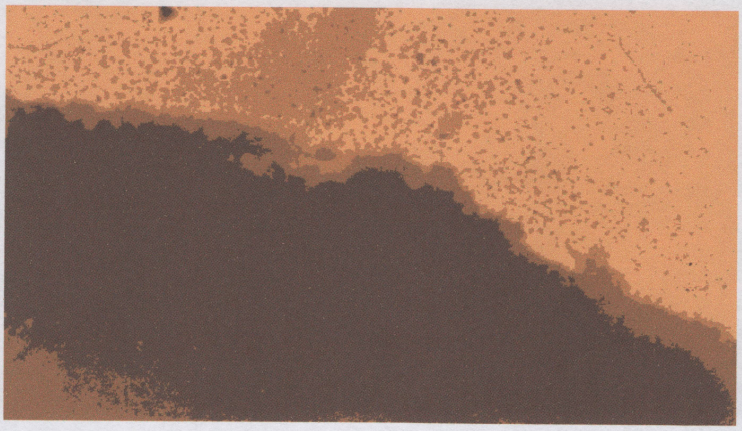

Fig. 13a: Granular particles dispersing from a *mountain* of cytoplasmic granules from which the enclosing growth sphere wall had lysed. Mag x 400.

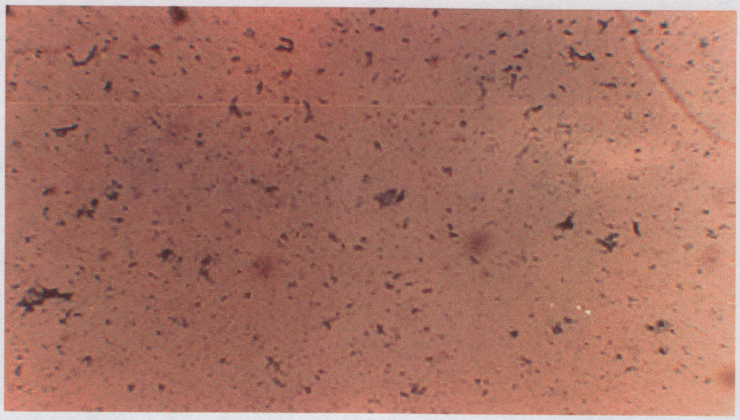

Fig. 13b: Granular particles unstained (at the background) and dispersed small clusters of particles. Mag x 400.

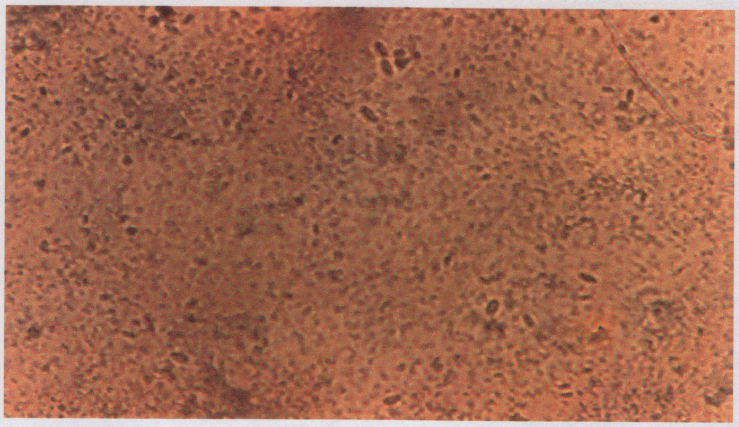

Fig. 13c: Enlarging granular particles. Mag x 400.

Yeasts induction sequence:	Spore swelling ------> Cytoplasmic granulation----> cell wall lysis--------> granular units disperse ------> protoplasts ------>yeast morphology evolvement -----> proliferating yeast morphology
Yeastlike induction sequence:	Spore swelling ----> Cytoplasmic granulation----> cell wall thicken ----> bud initiation -----> multipolar budding
Pseudomycelia induction sequence:	Spore swelling ----> germ tube emergence----> elongation by additive cells ---->branching-----> blastospore formation
Mycelia induction sequence:	Spore swelling ------>germ tube emergence ----> cell septation and branching----> aerial hyphae and sporangia formation

Fig. 14: **Sequential development in *Dimorphomyces* morphology**

A study on nitrogen source utilization by strain C13, IMI W5132B led to the recommendation on the use of $(NH_4)_2S0_4$ for further biochemical work (*in press*). Most other N-sources yielded poorly or induced multiple morphologies, although excluding hyphae. An earlier study (Omoifo, 1996b) showed that in $(NH_4)_2S0_4$ - medium incubated at 37°C, IG was followed by lysis of cell wall. But this did not occur when the N-source was peptone. Rather, such cells bud multipolarly. Bud formation at 37°C has been attributed to weakness created around cell wall β – glucan thereafter the site was blown out and a more active synthesis of chitin and α - glucan gave it a spherical shape (Kanetsuna *et al.,* 1972). Since $(NH_4)_2S0_4$–broth did not induce daughter bud formation by growth spheres, as did peptone-broth at the same temperature regime (Omoifo, 1996b), one tended to think that complex N-source, possessed within its range of amino acids and peptides (Segel, 1976) specific factor(s) that could be incorporated into membrane phospholipid structure (Hossack and Rose, 1976), and this might be contributory to the retention of cell wall integrity and synthesis of daughter bud cell wall after thermally tempered lysozyme effect and mechanochemical structural arrangement that possibly allowed outflow of granular cytoplasm. It seemed that multipolar budding was only a transition phenomenon between IG with intrinsic GUs and exposed GUs of the TM and, hence, it was considered only an adjunct to the SSYT hypothesis. This theory needs examining.

The assumption that a transmembrane-pH-gradient was established during the growth of strain C13, IMI W5132B also implicated the occurrence of electrophysiological activities. Experiments need to be conducted on key ion conductivity and their flow rates through plasma membranes of the different morphologies as component factors of E_m with the aim of determining effects on DNA synthesis and mitotic activities while taking into consideration the effects of polyelectrolytes and ionic strength on electrostatic potentials in the microsystems.

Two constituent cellular membranes were deputed in buffered $(NH_4)_2S0_4$–broth, one in each of the biphasic growth: growth sphere and yeast-oriented cell. Commencement of replicate activity during IG required membrane depolarization; similarly, proton-substrate symport into protoplasts during yeast cell evolvement likely led to a depolarization of E_m and either process was reversibly electrogenic. It was necessary to know how and when the presumed DNA replication was triggered off in the growth sphere and when stimulated, mitogenesis that led to yeast budding. If we assumed that the sporangiospore was the interphase in the life cycle of this eukaryotic microorganism, then it was necessary to know how mechanisms were switched on in their determined directions, or oriented, in direction and space, cytoplasmic vesicles for cell wall biosynthesis (Bartnicki-Garcia, 1987).

In one study (*in press*), we saw that the morphological expression of strain C13, IMI W5132B in low-level glucose broth was the YM but this shifted to PM at high-level substrate. Since the only difference was substrate level, it seemed reasonable to conclude that the originating cells of YM and pH had the same potential level for synchronous DNA replication and hence granulation capability. But high-level substrate imposed osmotic stress on ligand-receptor relationship. How this influenced E_m component of the electromotive force, need examining. Yet we saw multilaterally borne blastospores on each elongated cellular/ tubular unit of the PM, an indication of formation of daughter cells in a different electrophysio-morphological state, though when released, they were non-budding. This appeared to be a genetic adjustment derived from the transmembrane-pH- gradient: mitogenic nerve of our proposal, suited in the presence of reduced water activity. Hence this morphological expression was viewed as an extension of the SSYT hypothesis.

C. CELLULAR METABOLISM

i. A challenging exploration

Central to the SSYT hypothesis was the occurrence of a population with transient morphology. The granular particles, that is, cytoplasmic granules released following growth sphere wall lyses in the chemically defined medium with glucose as the only preformed organic source, became the protoplast from which yeast morphology evolved. When strain C12, IMI W5132A was used in similar medium, yeast cells were not induced. Morphologies induced were septate hyphae and various types of conidia including arthric, holothallic, and holoblastic, in addition to the population of TM. However, yeast cells were induced alongside the aforementioned morphologies when a growth factor like inositol, thymine or uracil was incorporated into the medium of growth (Omoifo, 1997). The growth pattern of strain C12, IMI W5132A has been derived. Observations indicated that the general mechanisms adduced for the sporangiospore-yeast transformation of strain C13, IMI W5132B were also involved in that of strain C12, IMI W5132A. However, the induction of yeast morphology in the absence of these growth factors in malate-, maltose- or

galactose-substrated multiionic broths (Omoifo, 1997; Fig. 15,16 and 17), posed a challenge to the appreciation of metabolic routes utilized in the physiology of strain C12, IMI W5132A. We hereby reassess the growth of this strain with a view to elaborating inherent concepts that the behaviors of strain C12, IMI W5132A permit within the experimental scope and further attempt to elucidate fundamental mechanisms for the transformation process.

ii. Observations suggesting sequential transformation: transmembrane- pH- gradient relationship

Synthetic broth used for cultivation of strain C12, IMI W5132A had the same type and quantity of elements as that used for yeast cell induction of strain C13, IMI W5132B (Omoifo, 1996b) except for the concentration of the buffer species, Na_2HP0_4: citrate, pH 3.5. Because of their nature, conjugant in solution provide effective buffer capacity; this presumably occurred during the growth of strain C13, IMI W5132B at pH 4.5 and hence the biphasic pH- profile observed during its growth was deemed to reflect proton flux through the growth sphere semipermeable membrane driven by transmembrane-pH-gradient (Omoifo, 1996b). If this was assumed the basic principles of Mitchell's (1967) chemiosmotic theory, which permit electrogenic energy transductions through biomembranes, were effective during isotropic growth of sporangiospores of strain C13, IMI W5132B.

Biomembranes have specific-cation transport systems (Jain, 1972; Zubay *et al.*, 1995). For instance, Na^+, K^+- ATPase, which is found in the higher eukaryotes, is an integral part of biomembranes and is responsible for the Na^+, K^+ oppositely coupled flux through the membrane (Tonomua, 1986; Lingrel and Kuntzweiler, 1994). K^+ ion is also exchanged for H^+ (Conway and O'Malley, 1946; Rothstein and Enns 1946, Pena, 1975). The influx of K^+ would lead to neutralization of the negative charge of cellular anions (Rothstein, 1960) thus increasing the intracellular pH (Pena, 1975; Harold, 1977; Camacho *et al.,* 1981). Since there was acidification of the bulk medium during the isotropic growth of strain C13, IMI W5132B, perhaps there was intracellular accumulation of K^+ leading to an increase in intracellular pH a situation that activates DNA synthesis, increase of cofactor- enzyme activities and protein synthesis (Jain, 1972; Harold, 1977; Rozengurt, 1986). From these activities, we suspect cytoplasmic granulation occurred. When growth sphere wall lysed as a result of thermodynamic equilibration on broth sides of the wall, which as well allowed lysozyme activities (Muarel and Douzou, 1978), the cytoplasmic granules or primordial biological units were thus released into the growth medium (Fig. 4) and thereafter dispersed (Fig. 5, 13a,b). The primordial biological units were observed to be in constant motion (13c). From these terminal budding yeast cells emerged (Fig. 6,10).

Similar morphologies including growth sphere, cytoplasmic granulation, cell wall lyses and release of cytoplasmic units, dispersal of primordial biological units and their restiveness, as well as matured terminal budding yeast cells (Fig. 15-17) were observed in the transformation process for strain C12, IMI W5132A. Thus the SSYT process enunciated for strain C13, IMI W5132B in section B was also adduced for strain C12, IMI W5132A.

iii. Possible mechanisms for stimulating DNA synthesis

The cell cycle is regarded as the sequence of changes involving DNA replication during the life span of a cell, from one mitotic phase which gives it its origin, to another from which it divides into daughter cells. This is conveniently divided into stages, from cell division G_o G_1, to *S* (DNA synthesis) to nuclear division to cell division. In *S. cerevisiae* this entails budding initiated before commencement of mitosis, but daughter bud secede only after one nucleus has migrated into the bud (Fincham *et al.,* 1979). In *Schizosaccharomyces pombe,* this synthetic period is also well defined although the *S* period is very short (Mitchison, 1973). The cell-nuclear relationship is not very well defined in filamentous fungi where independent nuclear (mitotic) cycles occur, as in coenocytic hyphae (Fincham *et al.,* 1979). Considering the fact that in surface cultures sporangiospores were the beginning and the end of the cell growth cycle of our dimorphic strains, then the sporangiospore, a product of asexual reproduction, was at the G_o stage, when the cell ceased all division. G_1 phase was thought to occur following inoculation of, and exogenous swelling of the sporangiospore. What followed was enlargement of the growth sphere (Omoifo, 1996b). This occurred during the first phase of the biphasic pH-profile, when there was further acidification of the growth medium, with an apposite intracellular pH increase and enhanced K^+ uptake (Ryan and Ryan, 1972; Borst-Powell, 1977; Camacho *et al.,* 1981).

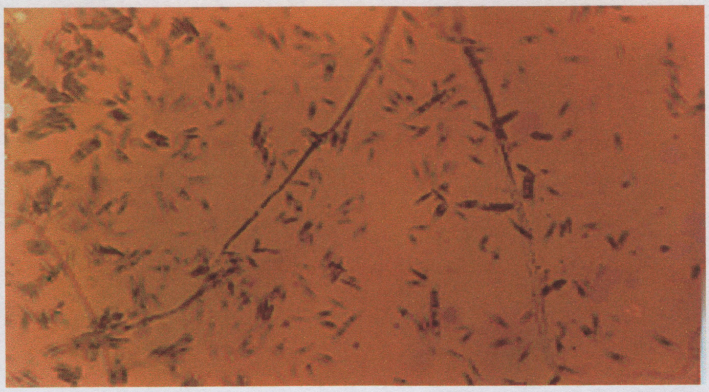

Fig. 15: Induced yeast cells and mycelia filaments of strain C12, IMI W5132A in malic acid substrate broth; mag. x 400. Growth conditions were as stated in the non-buffered medium of Omoifo (1997).

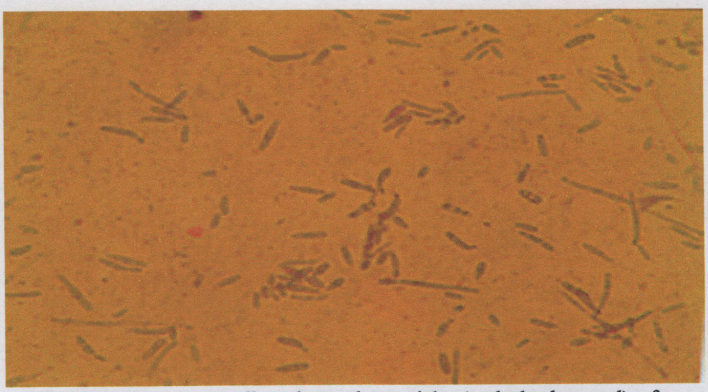

Fig. 16: Induced yeast cells and granular particles (at the background), of strain C12, IMI W5132A in maltose substrate broth; mag. x 400. Growth conditions were as stated in the non-buffered medium of Omoifo (1997).

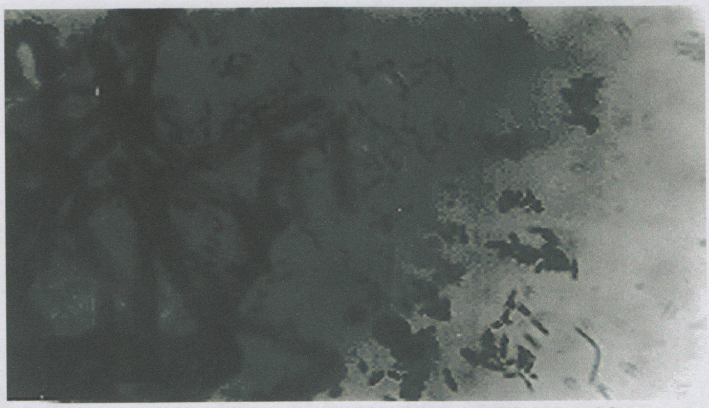

Fig. 17: Yeast cells, septate hyphae, and holoblastic conidia induced from sporangiospores of strain C12 IMI W5132A in galactose substrate broth; mag x 400. Growth conditions were as stated in the non-buffered medium of Omoifo (1997).

Furthermore, K^+ uptake could activate processes leading to intracellular differentiation. Chalazonitis and Fischbach (1980) imposed a K^+ concentration on neural cytoplasm and from microscopic cell counting exercise, autoradiography and laspe cinematography they found no increase in total cell number, yet neurons increased by 25%, in spite of the fact that there was no cell division. This increase was attributed to morphologic differentiation of the neurons.

The influx of K^+ into the intracellular medium, which was coupled to the efflux of Na^+, was under drive of a gradient potential, in which case, it was electrogenic (Dixon and Hopkin, 1980; Tonomura; 1986; Lingrel and Kuntzweiler, 1994). The work of Chalazonitis and Fischbach (1980) also showed that on challenge of ganglion neurons with bulk medium high- K^+, a depolarization of membrane potential, E_m, occurred. Control E_m = -53 mV, challenge E_m = - 22± I mV. However, it was not K^+ *per se* but the specific reduction of the intracellular concentration of Na^+, which caused the depolarization. The effect here was that the fall in E_m would lead to changes in molecular events. Cone (1985) monitored E_m of Chinese hamster ovary cell as well as labeled thimidine incorporation and DNA synthesis recovery, and found that a fall in E_m to –10mV was necessary for optimum DNA synthesis. Since inoculates of strain C12, IMI W5132A and strain C13, IMI W5132B were seeded into broths of high- level K^+, 3.67mM KH_2P0_4 + 2.89mM K_2HP0_4 (Omoifo, 1996b, 1997), which was a challenge, an electrochemical gradient was perhaps created across the biomembrane of the isotropically growing cells, and this also implied membrane depolarization of which the consequence was DNA synthesis. Similar to the non-dividing embryonic ganglion neurons (Chalazonitis and Fischbach, 1980), observation showed that the enlarging growth spheres of the dimorphic strains did not divide. Further still, like the morphologically–differentiated non-dividing cell embryonic chick dorsal root ganglion neurons, differentiation into cytoplasmic granules within the growth sphere envelope was conspicuous.

The pH-profile in the growth of strain C13, IMI W5132B showed that there was sustained and increasing negative magnitude from the bulk medium acid pH, phase I, which was interpreted as sustained uptake and accumulation of K^+ in the intracellular medium as well as continued depolarization of membrane potential, E_m, within the first 60h of growth. Cone's (1985) data on E_m - mitosis relationship showed that a high E_m of –75mV imposed mitotic blockage on CHO cells but this was after a prior blockage of DNA synthesis, albeit at a lower E_m level. Since we have presumed a high depreciation of E_m for strain C13, IMI W5132B, the level attained might be well below the threshold for blockage of DNA synthesis. If this was assumed, then mitosis did not occur during the isotropic growth phase of our dimorphic strains. But DNA synthesis did.

Thus, the isotropically growing cell was at the *S* phase of the cell division cycle, *cdc.* In eukaryotes, DNA polymerase- α, a multisubunit enzyme complex with a catalytic subunit that is in close association with a DNA primase that enables it accomplish its function, is the major replicate enzyme (Albert 1987, Abeles *et al.,* 1992; Aktipis, 1997; Fry and Loeb, 1986; McGilvery, 1983; Zubay, 1995). Other holoenzymes include DNA polymerase - β, δ and γ. Polymerase- γ is located exclusively in the mitochondria and presumably responsible for mitochondria replication (Abeles *et al.,* 1992; Aktipis, 1997). However, several authors including Fry and Loeb (1985) and Voet and Voet (1995) note that like the nuclei-bound α – and β – polymerases, a catalytically indistinguishable polymerase of the γ - type was also located in the nucleus in eukaryotes.

A possibility existed that the aforementioned polymerase species occurred in the isotropically growing cells, which were logically deputed to be at the *S* -phase of the *cdc,* in our study. These species have different requirements for metal activators. Fry and Loeb (1986) pointed out that the KB cell polymerase-α, which possessed multiple metal binding sites, preferred Mg^{2+} to Mn^{2+} which is an ion that was seven times less efficient as a metal activator. The preferred activator, they note, was required for nucleic acid: α – polymerase interaction, an event that was independent of the DNA polymerization process. On the other hand, Mn^{2+} was the preferred activator for γ – polymerase, its activity being enhanced in the presence of high levels of K^+ and phosphate, while reactivity of β – polymerase was suppressed in the presence of phosphate (Fry and Loeb, 1986). Since we have imputed an intracellular alkaline pH (preferable for polymerase activity), and with the exogenous supply of copious phosphate and Mn^{2+} in the cultivation medium, conditions were probably created for γ-polymerase activation.

Since polymerase- γ was responsible for mitochondria DNA replication (Fry and Loeb, 1986; Voet and Voet, 1995), perhaps one could attribute similar role to it in the nucleus, provided that the physicochemical and physiological conditions were right. Polymerase- γ participated in the initiation of DNA replication in prokaryotes like *E. coli* (Aktipis, 1997). Although the primary synthetic enzymes in eukaryotes are

polymerase - α and δ, it appeared that in a suitable environment polymerase- γ would form an enhanced profile with polymerase δ, thereby effecting replication. Thus, it was suspected that the priming and initiation of DNA replication, which presumably occurred at $G \rightarrow S$ transition stage of the *cdc*, involved DNA polymerase γ and its composite subunit proteins that would load onto an autonomously replicating complex, ARC, for subsequent initiation of DNA replication (Kearsey, 1987; Stillman, 1994). If these were assumed, then molecular relationships would perhaps be different from the lagging strand chromosomal DNA replication, as occurred in yeasts (Aktipis 1977; Fry and Loeb, 1986; Voet and Voet, 1995), with a possible morphologic amplification of such differences.

Hartwell and coworkers (1974) showed that bud initiation in *S. cerevisiae* started at *S*- period and that daughter bud separated from mother cell only after nuclear division and lodgement of the compliment chromosomes into the daughter bud and, hence progression of the *cdc*. Our work on the dimorphic strains showed that the proliferate phase, that is, yeast cells, spatially and morphologically distanced from the growth sphere morphology which, as we have argued, was at the *S*-period; its cytoplasmic granules were exposed to the growth medium after lyses of the growth sphere envelope, an effect presently attributed to electrostatic lysozyme activity on cell wall (Omoifo, 1996b), as a result of structural disorganization and destruction of the bimolecular lipid membrane associated with all eukaryotic cells (Jain, 1972).

The minute sizes and undefined shapes of the cytoplasmic granules or primordial units (Fig. 13a, b, c) released sharply contrasted with the commencement sporangiospore morphology (Fig. 3). In order to prove that the granular particles were not artifacts, a simple experiment was performed. Since a replicating specific DNA polymerase, and in this case polymerase γ species, would copy exactly the genetic material that produced it, the granular particles copiously produced at 15^{o}C were harvested and washed in sterile distilled water by centrifugation and thereafter cultivated in solid glucose-yeast extract-peptone or glucose multiionic medium surface cultures. Mould growths with similar characteristic features of strains C12, W5132A and C13, 5132B were obtained.

iv. Possible mechanism for establishment of physiological processes

Unlike what obtains in the *S. cerevisiae cdc*, the presumed *S* -phase, that is, the growth sphere morphology, was not accompanied by bud initiation. The ever- changing sizes and subsequent attainment of a regular form by individual primordial units of the population of TM indicated the occurrence of growth in the buffered multiionic broth. A tender biomembrane appeared to constitute the boundary of each entity. The entities were probably protoplasts, which could be globose (Fig 18). They were copiously produced at the inflexion point, 60h after inoculation of sporangiospores, in the biphasic pH- profile whereby a reversal of direction of the pH plot occurred, although the medium, still within the acidic zone, was obtained (Omoifo, 1996b). Thus, the electrogenic transductive activities were deemed to have continued through the presumed protoplasmic membrane and this, possibly, led to proton-substrate symport (Mitchell, 1967). The stoichiometric availability of substrate (West and Mitchell, 1972, 1973; Serrano, 1977; Eddy *et al.,* 1978; Alderman and Hofer, 1981) would then make carbon-energy source available for physiological utilization. This, of necessity entailed the construction of mRNA which would transcribe inherent information in the previously replicated trait-bearing DNA essential for building specific structural proteins for the ultimate utilization of the symported glucose in the case of strain C13, IMI W5132B (Omoifo, 1996b) and maltose, the preferred sugar for strain C12, IMI W5132A (Omoifo, 1997) transformation.

Thus in our buffered multiionic system, we suspected that the main physiological activity accompanying the *S* -period, DNA replication, after lyses of the growth sphere envelope and transductive electrogenic transport into primordial protoplasts, was transcription. The progressive increase in size of the primordial protoplasts was perceived as resulting from inherent construction and expansion of cytoskeletal structures including vacuoles, mitochondria, endoplasmic reticulum, ribosome, microtubules, lipid-containing granules in an apparently anaerobic environment. Reference was made above to the conversion of primordial units to mould growth in aerobic cultures. The protoplast, therefore, was considered a neoplastic unit that could be committed to specific physiological and, or morphological expression depending on the environmental condition.

Carbonell *et al*. (1973) presented results on the regeneration of cell walls by protoplasts previously derived from yeast cells of *Histoplasma capsulatum*, a dimorphic fungus. Centripetal invagination divided the protoplast into several masses; fibers appeared at the sites of invagination and later covered the surfaces of the cell membrane thus forming the first deposition of the cell wall, thereafter became interwoven within other wall components. These masses disengaged, producing yeast forms, which became terminal budding.

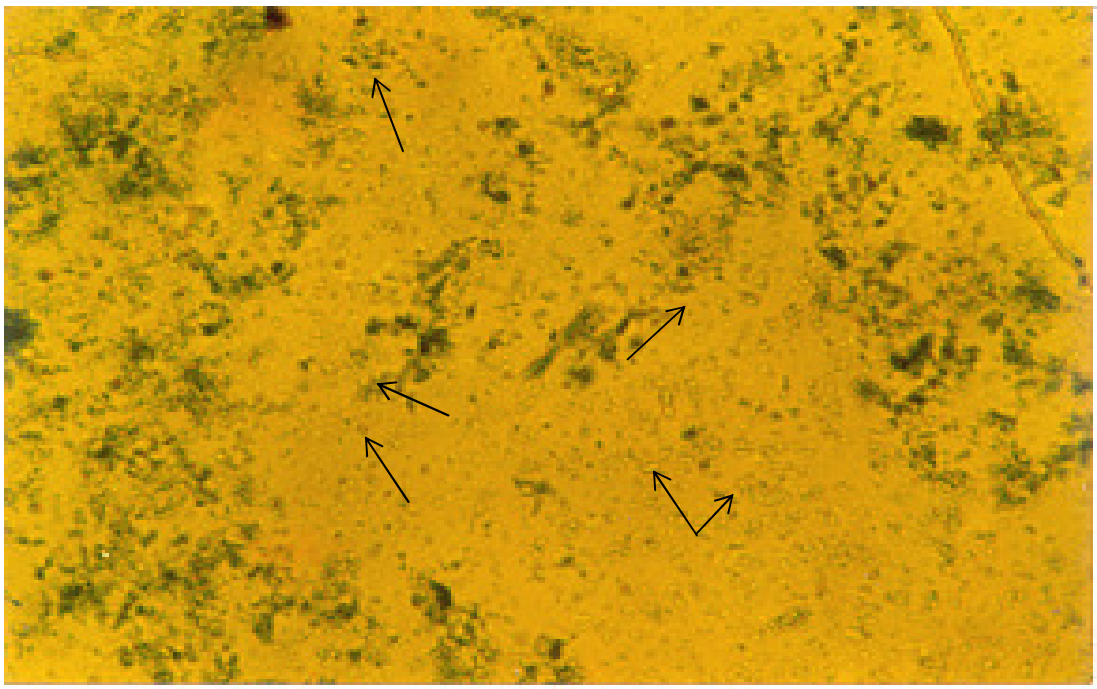

Fig. 19: Observe the enlarging particles (arrows) of strain C12, IMI W5132A with internal dimensions. These were thought to be protoplasts; mag. x 400.

On the other hand, multinucleated protoplasts without invagination, regenerated cell wall at the periphery, then became polarized thereafter producing tubular septate hyphae. The *H. capsulatum* yeast phase fibers were glucan, which was more abundant, and chitin, which according to Cabib (1975), is mainly found at the site of budding of *S. cerevisiae*. But the fiber of the hyphal phase of *H. capsulatum* was chitin.

Although the various anamorphic states, including the transient morphologies of strain C12, IMI W5132A or strain C13, IMI W5132B were viewed at x400 mag., the presumed protoplasts, in contrast to the regenerated protoplasts of *H. capsulatum,* were never observed to be invaginated in liquid or solid medium. It was our view that the individual protoplasts converted to yeast morphology on the one hand, and hyphal morphology on the other hand, depending on the environmental conditions.

The scheme in fig. 19 indicated that the common substrate, glucose, led to the construction of different cell wall structural components from which two distinct morphologies derived. In the presence of copious oxygen, presumably glucose in the cytosol was converted to glucose 6-phosphate from which activated glucosamine was derived and this was converted at the growing tip to chitin, polymerized into thin and slender- fibers- in-bundles, an action catalyzed by chitin synthetase, a zymogenically transported enzyme thereafter proteolyzed for activity. Simultaneously, and as a result of Pasteur effect, the partitioned glucose 6-phosphate was metabolically processed through oxidative phosphorylation. On the other hand, the cell wall structural material of the yeast morphology was derived after transmutation of glucose 6-phosplate to glucose 1-phosphate by the enzyme phosphoglucomutase; from this was derived activated glucose which was converted to glucan thereafter polymerized in thick microfibrils of varying length by the enzyme, glucan synthase. Since this transformative process presumably occurred in the buffered miltiionic broth considered an anaerobic batch culture, the yeast phase of strain C13, IMI W5132B possibly derived metabolic energy through the glycolytic pathway. Similar view was held for the transformative process for strain C12, IMI W5132A in the multiionic broth although it had its specific requirement for pH 3.5 and disaccharide-substrate, maltose.

v. Possible mechanism for cell division cycle

It has been argued (above) that mitosis did not occur during the cell's isotropic growth phase except DNA replication, which morphologically yielded cytoplasmic granules in the growth sphere compact. After lysozyme destruction of the growth sphere envelope, individual units emerged as protoplasts. Proton-substrate symport would make available within the protoplasts carbon energy source, which became partitioned and thereafter dissimilated through the glycolytic, and cell wall biogenetic pathways that cooperatively led to the yeast morphology. Since the extra cellular medium of growth of strain C13, IMI W5132B tended toward alkalinity, second phase of the pH-profile, purportedly vectoreal, an indication of the continuation of the transmembrane-pH- gradient, but in this case in the reverse order, which also enabled the stoichiometric electrogenic antiport activity of membrane bound Na^+, K^+- ATPase (Dawes, 1986; Tonomura, 1986; Voet and Voet, 1995) that apparently permitted the pumping of K^+ out and Na^+ into the evolving yeast cell, then the intracellular accumulation of Na^+ could initiate the mitotic event (Cone, 1985) by modifying and or increasing the electrostatic potential of the polyionic atmosphere surrounding the negatively charged polyvalent chromatides (Maurel and Douzou, 1978). This would then lead to chromatide equatorialization, segregation, eventual separation into daughter nuclei and subsequent nuclear membrane formation (Fristrom and Spieth, 1980). The daughter nucleus would thus migrate into the daughter bud.

Intracellular conditions could produce modifications of biochemical nature, which might alter molecular and ionic species. The biphasic pH-profile and the perceived transport mechanisms have enabled us to focus on K^+ and Na^+ as key elements that accumulated intracellular respectively in phase I and phase II but in two distinct morphological forms: growth sphere and evolving yeast. Each would however attract other cooperative ionic species. Since the different polymerase species could be found in the same eukaryotic nucleus (Aktipis, 1997), strain C13, IMI W5132B inclusive, the type activated under the two sets of intracellular conditions would probably differ. One logic of reasoning above have it that polymerase- γ with a requirement for high K^+ and phosphate levels, and Mn^{2+} as metal activator, was used for synchronous DNA replication. We also suspected that with the possible intracellular accumulation of Na^+, Mg^{2+} as metal activator (Fry and Loeb, 1986) at the second phase of the pH- profile, and this became part of the polycationic environment for optimum polyanionic polymerase- α electrostatic interaction (Maurel and Douzou, 1978) at the genome level then, this DNA enzyme could become active. If polymerase-α was activated, it could work in close association with polymerase-δ so as to ensure high fidelity DNA

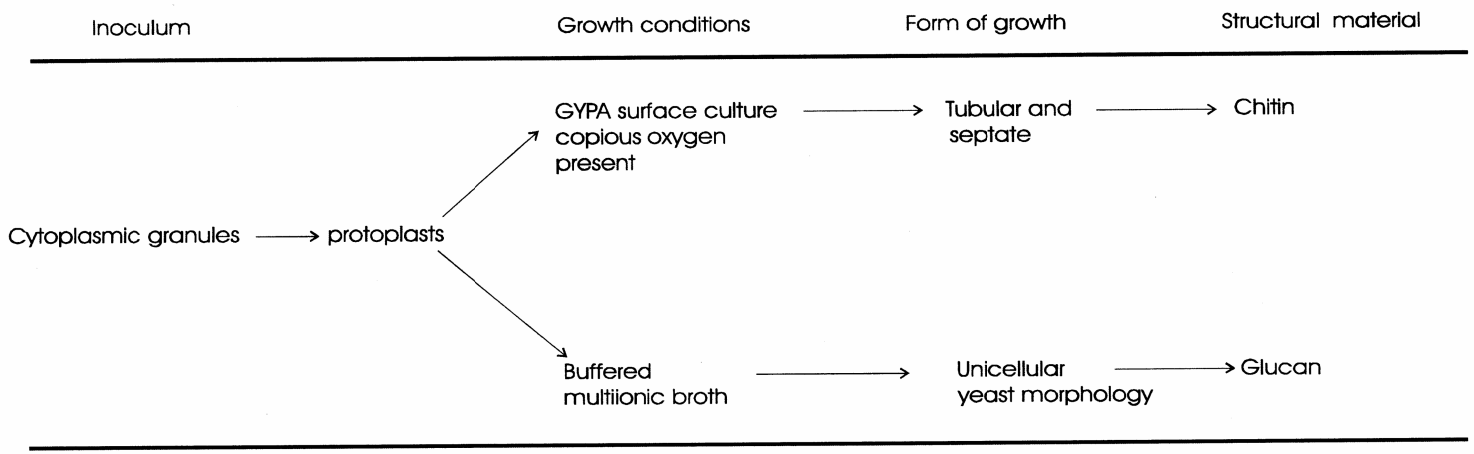

Fig. 20: Scheme for the morphological expression of strain C13, IMI W5132B. Cytoplasmic granules, released after growth sphere wall lysis, were copiously produced in glucose - substrated multiionic buffered broth, pH 4.5 incubated at 15^{0}C, ambient.

replication (Aktipis, 1997) at the S- phase after formation of the yeast morphology, which became so conspicuous at the second phase of the pH- profile in our study (Omoifo, 1996b).

The illustration by Cabib (1975) showed that the separation of *S. cerevisiae* bud started with formation of a 'neck' between mother and daughter bud. This neck region thickened, followed by centripetal growth of a thin disk of chitin fibers, which constituted the primary septum. The disk separated the plasma membrane of mother and daughter cell. A secondary septum made up of glucan fibers and mannan interstitial materials could develop between the disk and membrane on either side and in continuation of the primary cell wall. The bud finally detached, the disk remaining with the mother cell. Continued mitotic activity and separation would lead to proliferation of a cell line. Since bud formation- separation-bud formation effect was observed during the growth of our dimorphic strains, then the anatomical detail of *S. cerevisae* propagation was probably true in this case. Figure 20, 21 showed clusters of terminal budding yeast cells that originated from cytoplasmic granules/protoplasts of strain C12, IMI W5132BA and strain C13, IMI W5132B cultivated respectively in maltose-substrated, uracil–incorporated and glucose-substrated multiionic broths.

Therefore we suspected that the normal *cdc,* with $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$ periods, attributable to *S. cerevisiae* would be approximated by the evolved yeast morphology of our dimorphic strains. This was shown in the conjugate recognized segment of the heteromorphologic conjugal-*cdc* (*hc-cdc*), in fig. 22. It was pertinent to point out that *Sch. pombe* which has a distinct budding pattern, binary fusion, shares similar *cdc* with *S. cerevisiae,* although its S-period is shorter (Mitchision, 1973) but DNA polymerase- α is the main replicate enzyme involved in these two yeasts which are separately holomorphologic in their life cycles. On the other hand, strain C12, IMI W5132A and strain C13, IMI W5132B are heteromorphologic although strain C13, IMI W5132B proved to be more pleomorphic. This was depicted in fig. 23, 24.

Two *S*- periods separated by two distinct morphologies, in time and space, and each perceivably with a distinct core polymerase species were implicated in the SSYT process. This contrasted with the earlier suggestion that mitotic activity occurred within the growth sphere i. e. during isotropic growth (Omoifo, 1996b). One further question that needed to be
examined was this: could the same DNA biosynthetic enzymes be said to be involved in the sporangiospore- yeast-like transformation process?

Observation showed that isotropic growth and cytoplasmic granulation, early features in the SSYT process, also characterized the sporangiospore-yeast-like process. Yeast-like cells were enlarged growth spheres (globose) that became multipolar budding. Daughter buds were also globose, varied in size but cytoplasmic contents were granular as that of the mother cell. Further observation showed that multipolar budding occurred at elevated temp when organic compound, peptone, was the nitrogen source. Budding of yeast-like cells occurred mainly at the second phase of the pH-profile (Omoifo, 1996b). This implied that multipolar budding was analogous, in time frame, to the terminal yeast budding. Remarkably, in the former, the growth sphere envelope did not lyse, cytoplasmic units were not released and protoplasts were not formed. However, the yeast-like cell envelope could lyse due to pH variation (Omoifo, 1996b). When lyses occurred, protoplasts were observed and from these yeast cells emerged.

These interesting observations led to the perceptive view that mitotic activity could not be streamlined until the original cell envelope, that is, sporangiosporal-turned- growth sphere envelop, was destroyed. Consequently, the γ-polymerase induced cytoplasmic units- turned protoplasts, with a plasma that could be stimulated or directed for proliferate progression in a specific phenotypic expression stimulated by a different genomic enzyme species, polymerase α, and this became established in a proton-substrate symported, Na^+- K^+ antiported and transmembrane-pH-gradient driven physiological relations. It was further thought that the mitotic event, DNA polymerase- α activated, did not occur within multipolar budding yeast-like cells, since the mother cell envelope did not lyse. Rather, after the initial electrogenically driven mechanisms, ATP generation by substrate level phosphorylation, which possibly called for enzyme isoforms adapted to thermal conditions, supplied high-energy bonds for the continuation of DNA replication. Thus there was a prolonged S-period. The preponderance of replicated units, each with a possible early protein complement, within the non-lysed growth sphere/mother cell migrated into the daughter buds. A scheme for the *cdc* of yeast-like cells of strain C13, IMI W5132B is presented in fig. 25. Perhaps polymerase- γ, or its associated protein could also activate processes that led to daughter bud maturation and separation. Table 3 listed the induced morphologies from sporangiospores of strain C13, IMI W5132B, possible transformative events and associated polymerase species.

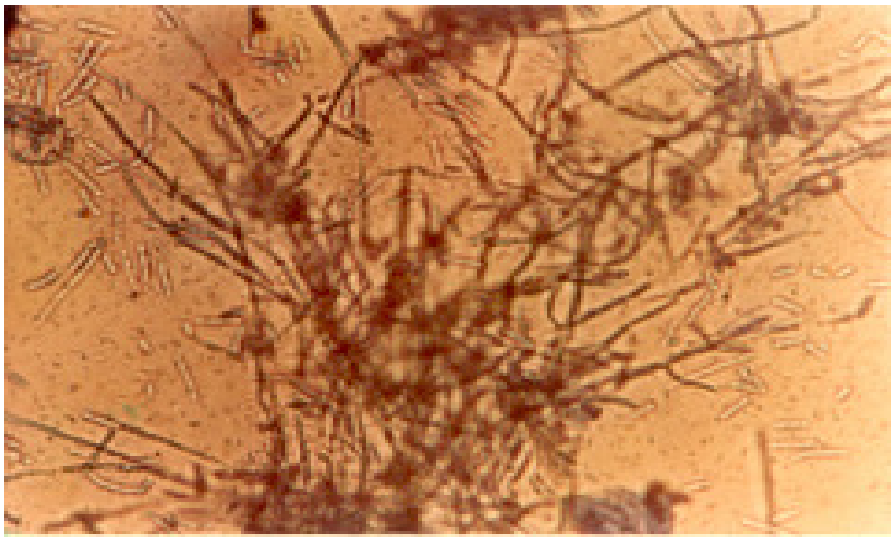

Fig. 21: Clusters of induced yeast cells of strain C12, IMI W5132A in maltose-substrated, uracil- supplemented multiionic-buffered broth, pH 3.5, after 3 days of growth at 20^{o}C ambient. Mag. x 400.

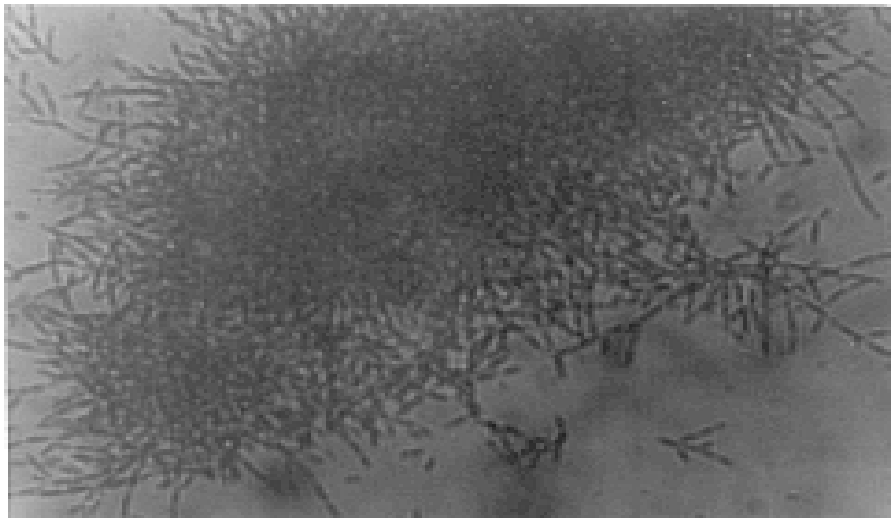

Fig. 22: Clusters of yeast cells induced from sporangiospores of strain C13, IMI W5132B in glucose substrated, multiionic buffered broth, pH 4.5, after 3 days of growth at 20^{o}C ambient. Mag. x 400.

Table 3: Classification of possible events during the transformation of sporangiospores of strain C13, IMI W5132B

Morphology Start	Morphology Final	Transformative event(s)	Complementary biochemical process	Active polymerase species
Sporangiospore	Growth sphere	Sphericity, metabolic initiation	Passive transport osmoregulation	
Growth sphere	Cytoplasmic granulation within non-lysed growth sphere	Synchronous DNA replication; growth sphere envelop lyses	Transmembrane- pH- gradient lysozyme activation; K^+- influx	δ, γ, β
Cytoplasmic granules	Protoplast	Transcription and translation of early proteins, organelles	H^+- glucose symport K^+-Na^+ antiport	δ, γ, β
Protoplast	yeast cell	Cell wall biogenesis; bud initiation; spindle plaque formation; mitosis	Glycolysis; glucan + mannan- protein synthesis	δ, α, β
Protoplast	Mycelia	Tubular cell wall biogenesis septation, nuclear division	Oxidative respiration chitin + galactomannan-protein synthesis	δ, α, β
Sporangiospore	mycelia	Tubular cell wall biogenesis nuclear division	Chitin +galactomannan- protein synthesis, oxidative phosphorylation	δ, α, β
Sporangiospore	pseudomycelium	Tubular cell wall biogenesis nuclear division blastospore formation and release	Chitin + galactomannan protein synthesis; mitotic arrest in blastospores catabolite repression	δ, α, β
Sporangiospore	yeast-like cell	Synchronous DNA replication; non-lysing of cell envelope; multipolar bud formation; daughter bud separation	Transmembrane-pH- gradient Na^+, K^+ antiport; H^+- glucose symport; glycolysis chitin + glucan biogenesis	δ, α, β

vi. Possible mechanism for glucose and oxygen effects

The fact that sporangiospores of strain C13, IMI W5132B converted to terminal budding yeast cells in glucose substrated multiionic broth while those of strain C12, IMI W5132A expressed as mycelia and conidia in similarly substrated medium indicated that a fundamental difference existed between the two strains. When fig. 22 is juxtaposed with fig. 24, it appeared that the 'decision' of the protoplast of strain C13, IMI W5132B to express the unicellular or tubular form occurred at G_2 of *cnh-cdc.* But these were at two different environments in which the major difference was the copious availability of atmospheric oxygen; this was required for oxidative metabolism. Thus, it seemed the availability of oxygen altered the process of transformation to the yeast form.

Since proteins were involved in either processes of transformation, the induction of specific genes was thus affected. It made sense to say that specific gene products were negatively affected in one process, in comparison with the other. This was considered in a similar manner with the negative control of β-galactosidase, an inducible enzyme for lactose utilization, in the presence of glucose (Jacob and Monod, 1961). In our study, glucose availability encouraged filamentous growth in air, in which case, there was respiratory metabolism. The same substrate induced yeast-forming enzymes that yield terminal budding yeast morphology in broth (*in press*). Gene regulation occurs at the level of transcription where an inducer prevents a repressor from binding to DNA thereby stimulating RNA polymerase to synthesize mRNA, which are subsequently translated to proteins. When glucose is present in lactose- substrated medium for the cultivation of *E. coli,* β - galactosidase synthesis is prevented. This is the glucose effect (Pastan and Adhya, 1976). The induction of yeast morphology from sporangiospores of strain C13, IMI W5132B cultivated in glucose-substrated multiionic broth was viewed as positively driven, an effect counteracted in surface cultures by the presence of copious oxygen. Thus, copious oxygen 'induced' filamentous growth. Perhaps, one could talk about 'oxygen effect'. The scheme in fig. 26 showed the possible reaction steps and enzymes exposed to the inhibitory effect of oxygen.

In a batch-grown microorganism, the ever-changing environment directly alters its physiology (Calcott, 1981). Michelson (1978) noted that non-enzymatic reactions that occur in respiratory systems under anaerobic conditions could lead to the generation of activated oxygen species including superoxide (0_2^-), hydroxyl radical (.0H) and dianion ($.0_2^{2-}$, or its undissociated form, H_20_2) which could provide the 'oxygen effect' in liquid medium. Superoxide is formed by a one- electron reduction of 0_2 by Fe^{2+}, CO_2^+, or Ni^{2+} when a complexing agent is present, the complexant being a hydrogen donor. Another factor that contributes to spontaneous dissimilation of superoxide in a buffered anaerobic environment is high-level phosphate availability (Michelson, 1978).

The multiionic broth used for yeast morphology induction of strain C13, IMI W5132B was buffered and time-lag study showed a biphasic pH-profile. It has been argued that this physiologically was tantamount to the establishment of a transmembrane - pH - gradient whereby H^+ influx with symported glucose into the intracellular medium occurred at the second phase of the pH-profile, when phosphate was also actively transported inward (Lehninger, 1975) from a high-level phosphate external bulk medium. Thus, conditions were probably set for non-enzymatic reactions, on the one hand, and biochemical interactions, on the other. The H^+ in the cytosol could play two different roles (i) modify the high protoplasmic pH level resulted from the earlier proton extrusion (1^{st} phase pH-profile) (ii) act as a reducing agent in the spontaneous reaction leading to superoxide (0_2^-) and H_20_2 production, i.e. activated oxygen species (AOS) or reactive oxygen intermediates (R01s). Fig 27 illustrated a scheme for the possible generation of R01s.

Between the releases of cytoplasmic granules, resulting from lyses of growth sphere envelope, and protoplast formation from which yeast morphology emerged, the intracellular medium was deemed to be neutral to alkaline. This condition favored the Fe^{3+} state (Awad, 1997). The entry of H^+ into the forming protoplast (tendency towards alkalinization of extracellular medium) would then lead to a reduction of Fe^{3+}, a situation that occurred under anaerobic conditions (Michelson, 1978) which also enhanced glycolytic breakdown of phosphate esterified glucose, glucose 6-phosphate, the end products being ethanol and ATP. It would appear that the formation of yeast cell wall by the protoplast was not directly dependent on the glycolytic process but the potential to convert glucose 6-phosphate to another key reaction product, glucose 1- phosphate. These divergent metabolic routes probably matter as the yeast form of *P. brasiliensis* contained a higher level of the enzyme phosphoglucomutase, Mg^{2+}- requiring, in comparison with the mycelia form (Kanetsuna and Carbonell, 1966). This could be important because sub optimal level of a gene product could possibly lead to non-expression of an ensuing phenotype.

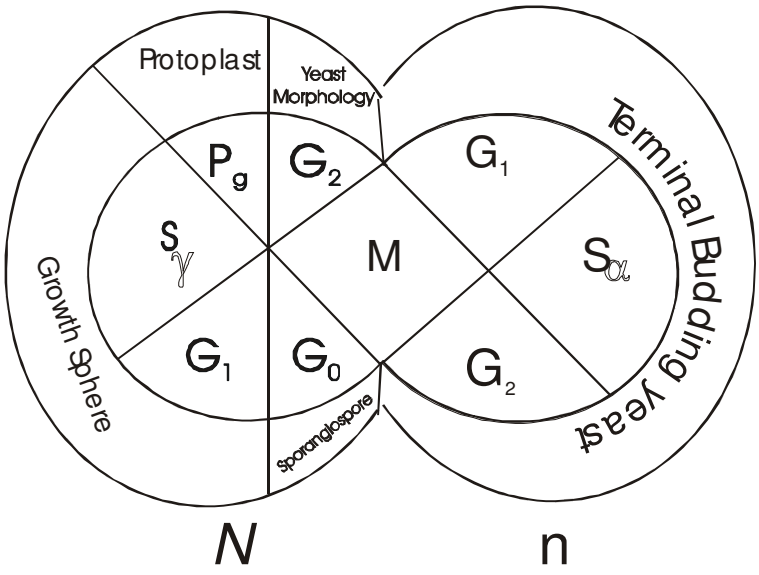

Fig. 23: Scheme illustrating possible heteromorphologic conjugal- cell division cycle (*hc-cdc)* for, and yeast cell induction from sporangiospores of, strain C13 IMI W5132B cultivated in glucose - substrated multiionic buffered broth, pH 4.5 at 20^{0}C. Two phases of the *hc-cdc* were shown: *N* = novel - *cdc*, n = normal - *cdc.* Representative subphases of *Nhc-cdc:* G_0 = quiscence, G_1 = isotropic growth, establishment of transmembrane - pH - gradient, K^+- modified intracellular medium; Sγ= γ- polymerase - committed synchronous DNA synthesis occurred during isodiametric growth; P_g = growth sphere wall lysed releasing cytoplasmic granules into the medium, multidimensional growth of cytoplasmic granules into protoplasts, anisotropic ion distribution, H^+- substrate symport; G_2 = glucose metabolism: glycolytic process, transmutation to inositide moiety and inositide cascade, activated substrate, yeast morphology, and mitogenic signals pathway evolved; *nhc-cdc* subphases: G_1= migration of daughter nucleus into daughter bud, separation of daughter bud; S_α= α - polymerase committed DNA replication; G_2= preparation for mitosis.

If the protoplast 'yeast potentiality' was sufficiently high in a certain condition, agonists of the genome system could stimulate transcription of genes subsequently translated to specific proteins that would activate biochemical reactions in a desired physiological process. Since membranes are dynamic structures adaptable for specific functions (Jain, 1972), the protoplast could then be subjected to the same thermodynamic parameters, stoichiometry of constituents, spatial occurrence and density of biocatalysts as occurred in *S. cerevisiae* (Seaston *et al.,* 1973; Soumoainen and Nurminen, 1973) and *Metschnikowia reukaufii* (Alderman and Hofer, 1981). Demand for biosynthetic products would be high at this primordial stage and exacerbated utilization of symported glucose via substrate level phosphorylation would appear to be the main process for energy supply. Apart from the enzymes of glycolysis and the pentose phosphate pathway, PPP, those for the yeast wall biosynthesis would probably include phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, and β-1, 3-glucan synthase. If all these enzymes were part of the intracellular constituents of protoplasts of strain C13, IMI W5132B, then the occurrence of terminal budding yeast morphology in glucose-substrated buffered multiionic broth could be accounted for. It however does not account for the fate of the non-enzymatically generated ROIs alluded to earlier.

R01s are toxic to life forms (Conn and Stumpf, 1976). For instance, superoxide spontaneously combines with the peroxides to form hydroxyl radicals and singlet oxygen

$$0_2^- + H_2\,0_2 \rightarrow H0^{\cdot} + {}^1\!0_2 + 0H^-$$

and these can cause DNA damage, lipid peroxidation or protein oxidation (Martini and Ursini, 1996). However, mechanisms have evolved in all aerobic and oxygen tolerant organisms (Conn and Stumpf, 1976; Fridovich, 1978; Michelson, 1978; Mcgilvery, 1983) as well as anaerobic bacteria (Michelson, 1978) to contend with this effect. It involves antioxidants like superoxide dismutase (SOD), glucose 6-phosphate dehydrogenase (G6PD, glutathione reductase/peroxidase system, which prevent oxidative injury (Martini and Ursini, 1996). For instance SOD destroys superoxide

$$2H^+ + 0_2^- + 0_2^- \rightarrow H_20_2 + 0_2$$

Manganese –requiring SOD has been found in the mitochondria and cytoplasm of the basidiomycetes *Pleurotus olearius* where the alternate oxidation and reduction of the metal enables the enzyme to carry out its functions.

$$\text{Mn (III) - SOD} + 0_2^- \rightarrow \text{Mn (II) -SOD} + 0_2$$
$$\text{Mn (II) - SOD} + 0_2^- \rightarrow \text{Mn (III) - SOD} + H_20_2$$

RO1s are also generated by the incomplete oxidation of oxygen to water during respiration (Michelson, 1978; Voet and Voet, 1995). It meant, therefore, that in liquid medium, two possibilities existed for the generation of RO1s: enzymatic reaction and non-enzymatic reaction. Apart from this, organisms have also evolved other ways of eliminating the toxic effect of RO1s. This could be by incorporating it into the metabolic process. For instance, the first step in the catabolism of the aromatic amino acid, tryptophan, involves the combination with the superoxide anion catalyzed by the heme-containing enzyme, tryptophan 2, 3 dioxygenase (Conn and Stumpf, 1976; Hayaishi, 1978; Coomes, 1997). The anion is also utilized in the paraphenylpyruvate and homogentisate reaction steps in tyrosine metabolism (Voet and Voet, 1995).

For strain C13, IMI W5132B the possibility for metabolic utilization of superoxide anion existed. In a survey to demonstrate the ability of this strain to utilize inorganic, organic and complex nitrogen sources, it was found that tryptophan, which requires superoxide anion for catabolic access to its indole ring, supported growth to the same significant level as L-cystine. HCl, cysteine, tyrosine, vitamin- free casein hydrolyate, $KN0_3$, methionine, adenine, lysine, peptone, DL- alanine, glycine, para-amino-n-butyric acid, leucine, DL-aspartic acid, L-histidine. HCL. H_20, L- proline and DL-serine although, optical density reading was significantly different from that of $(NH_4)_2S0_4$ and urea, which had higher values. Metabolism of many of these N-sources does not involve disruption of phenyl or pyrrole ring. But the superoxide anion utilized in the metabolism of tryptophan, or tyrosine could have been generated enzymatically or non-enzymatically as explained above. The predominant morphology induced in the N-sourced broths was terminal budding yeast form; of note was the occurrence of hyphal fragments, holoblastic conidia and

growth spheres in tryptophan sourced broths in contradistinction to the sole yeast morphology induced in $(NH_4)_2S0_4$ – and urea-sourced media.

vii. ***Possible mechanism for phosphofructokinase: phosphoglucomutase coupled activity***

In glycolysis, the activation of phosphofructokinase, PFK, causes a rise in fructose 1,6 bisphosphate, F1, 6 bisp, concentration a situation paralleled by an increase in glucose utilization (Lowry *et* al., 1964). The study of Dietzler *et al.* (1975) showed that in *E. coli* W4597 (K) the increased activity of PFK, which was the rate limiting step in glycolysis, caused a decrease in the intracellular levels of G6P, a covariant of fructose 6-phosphate F6P, and this also affected, in quantitative terms, glycogen synthesis. The good fit of data obtained from *in vitro* and *in vivo* experiments led Dietzler and colleagues to conclude viz (i) F1, 6 bisP covaried with G1P, (ii) G1P was the activator as well as substrate for ADP –glucose synthase (iii) the rate of glycogen synthesis was equivalent to the velocity of ADP- glucose synthesis and (iv) G1P was the compound which altered the reaction rate. It was further shown that a coordinate relationship existed, between the energy generating EMP pathway and energy utilizing pathway for glycogen synthesis.

The yeast PFK, the crossover point in the metabolic control of glycolysis (Wu, 1964; Betz and Chance 1965), generates biochemical oscillations for glycolytic intermediates along with other mechanisms involving feedback systems (Betz and Moore, 1965). Similar coordinated regulation of metabolism obtained with other physiological processes. Brown and Wittenberger (1971) showed that *Streptococcus feacalis* growing in anaerobic environment channeled glucose, in part, through EMP pathway and PPP. In their study, it was demonstrated that the EMP intermediate, F1, 6 bisP specifically inhibited 6-phosphogluconate dehydrogenase, 6PGD, although, the enzyme activity was not completely prevented. The 6PGD from other sources including bacteria, yeast and animals were similarly inhibited. This therefore showed that a coordinated regulation also occurred between EMP and PPP used for the generation of biosynthetic intermediates in organisms. In yeasts, this could be achieved by carefully adjusting the concentration of different cofactors including the ADP, AMP and ATP (Polakis and Bartley, 1966). For an organism growing in an anaerobic environment, there would appear to be a finely tuned interactive distribution and control of carbon substrates into the three pathways for energy generation, energy utilization (including cell wall biogenesis) and the generation of biosynthetic structures. Glucose 6-phosphate seems to be central to these branch routes. Fig. 28 is a perceptive view of such relationships.

In the first glycolytic reaction, the enzyme hexokinase transfers phosphoryl group from the nucleotide ATP, potentiated by Mg^{2+}, which also serves as feedback signal (Blair, 1970), to the 6C-0H--glycosyl metabolite. It is this compound that is distributed through the various metabolic routes. We note that Mg 2.0g/l was included in the medium preparation for the growth of our dimorphic strains (Omoifo, 1996b, 1997). Merrill and Pitot (1986) pointed out that enzyme inductions could also be stimulated by simple carbohydrates like glucose, pyruvate and glycerol which respectively induce glucokinase, pyruvate kinase and lypolitic enzymes. Hence we suggested that in the multiionic broths used for yeast cell induction from sporangiospores of strain C13, IMI W5132B, symported glucose in the protoplasts induced the glucokinase enzyme. The presence of this Mg^{2+}- requiring enzyme would therefore catalyze phosphorylation of the 0H group of C1 of the G6P molecule by ATP, thus forming glucose 1, 6 bis phosphate (G1, 6 bisP). This was shown in fig. 29. The presence of G1, 6 bisP, albeit in small quantities, was obligatorily required to activate PGM, the principal enzyme that kinetically converts G 6 P to G1P (Voet and Voet, 1995; Harris, 1997). Since PGM was detected in both yeast and mycelia forms of the dimorphic fungus *P. brasiliensis* (Kanetsuna and Carbonell, 1966), we inferred that phosphoglucokinase was present *in vivo.* The data of Kanetsuna and Carbonell (1966) showed that PGM content was upped by 68.94% when the mycelia form of the microorganism converted to yeast morphology, a possible development arising from phosphoglucokinase- phosphoglucomutase coupled activities in anaerobic conditions. If such activities were assumed for the dimorphic strain C13, IMI W5132B cultivated in buffered mulitiionic broth, then PGM would probably be raised from a base level to an induced high level. Thus potentiated the enzyme, which has a serine –0H group at the reactive end, could attack a phosphoryl group at C6 of G 1, 6 bisP to form a phosphoenzyme with the end products being G 1 P (Fig. 30i). This trigger could therefore signal a continuation of the phosphoenzyme-serine catalysis of G6P conversion to G1P (Fig. 30ii).

The point has been made that G1P covaried with F 1, 6 bisP. Therefore, increased activity of PFP which ensured a high level of F1, 6 bisP would also elevate the concentration of G1P, a metabolite in the energy consuming process of the cell. G1P is transformed to biopolymers by firstly activating the sugar: a reaction

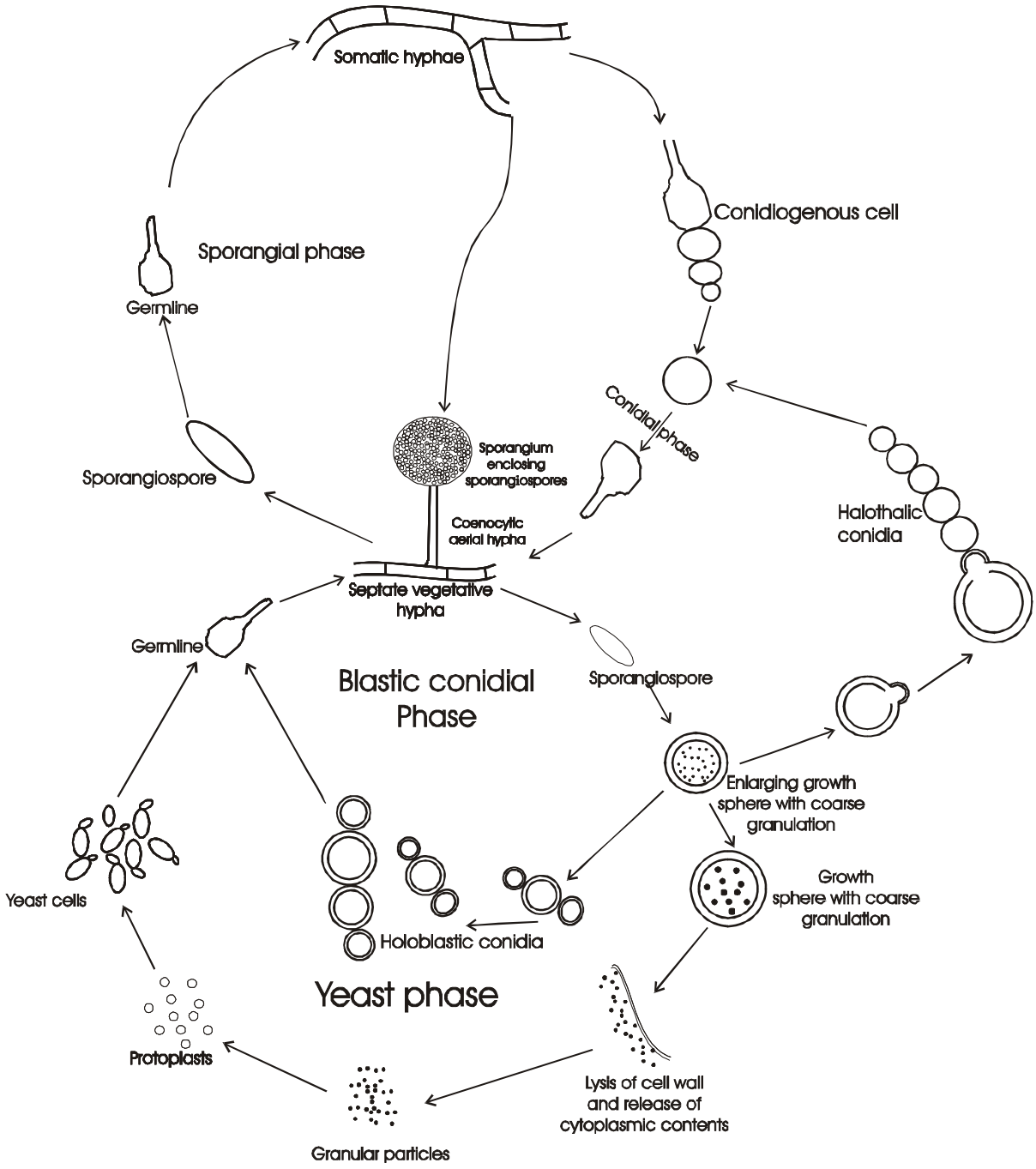

Fig. 24: Proposed life cycle of strain C 12, 1Ml W5132A

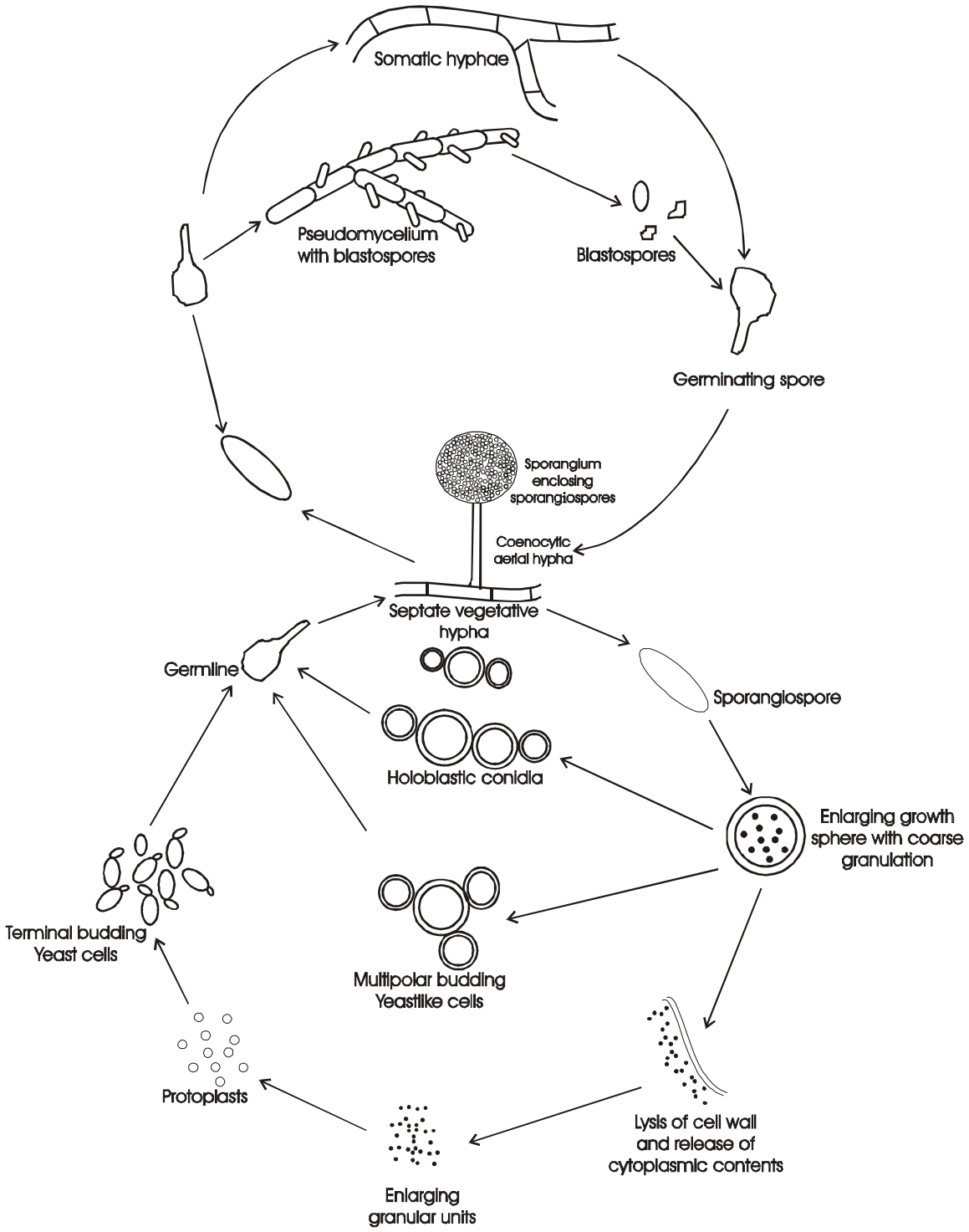

Fig. 25: Proposed life cycle of strain C 13, 1Ml W5132B

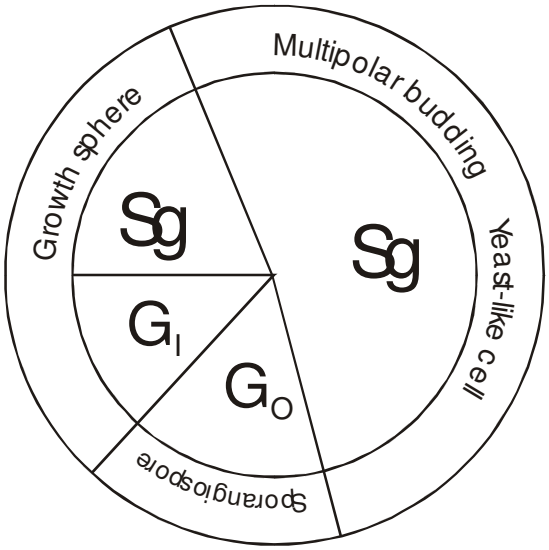

Fig. 26: Scheme illustrating possible cell division cycle, *cdc,* for, and yeastlike cell induction from sporangiospores of, strain C13, IMI W5132B cultivated in glucose - substrated multiionic buffered broth, pH 3.5, temp. 20^0 - 37^0C with peptone as the only source of nitrogen supply. The subphases were possibly similar to *n-cdc* in Fig. 109, except the multipolar budding yeastlike morphology in which there was a probable continuation of g - polymerase - committed synchronous DNA synthesis. By this scheme, daughter bud formation through blastic action was not accompanied by nuclear division as in mitosis.

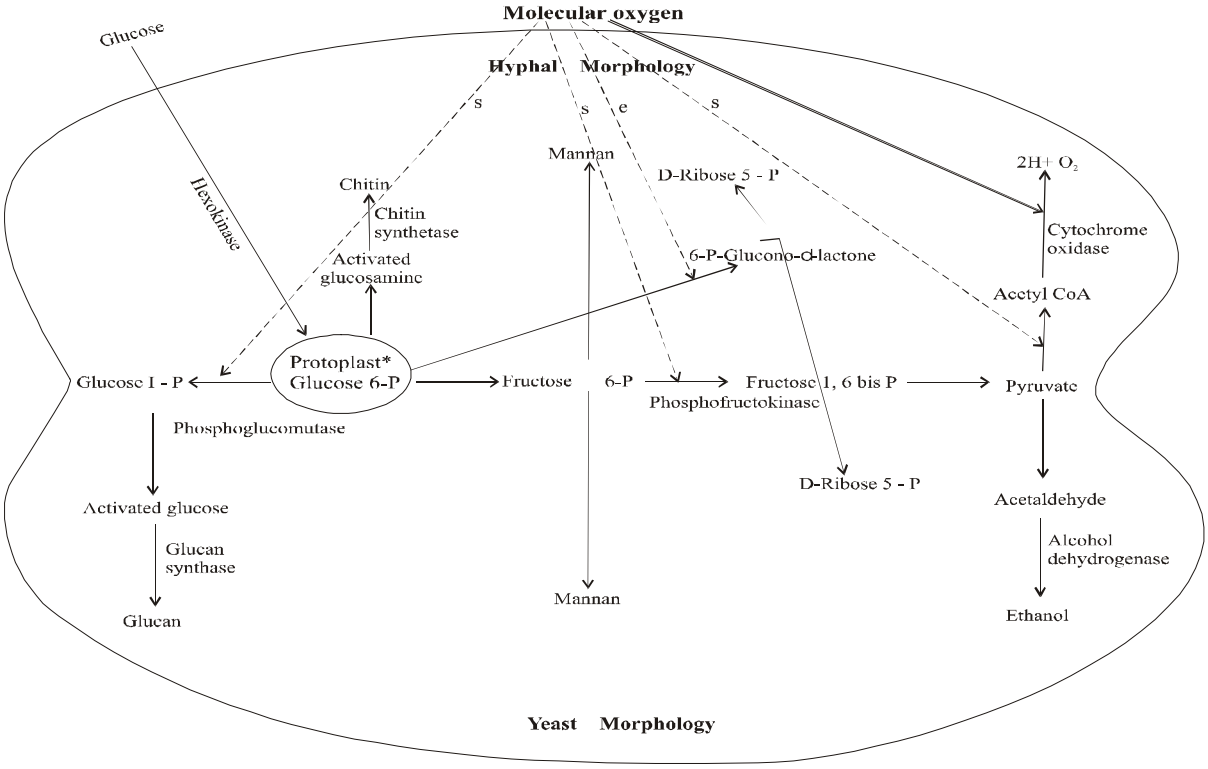

Fig. 27: Scheme indicating possible reaction steps where high oxygen intensity exerts control during morphogenetic conversion of cytoplasmic granules/protoplasts to hyphal or yeast (terminal budding) morphology.

- ----→ Control step as a result of oxidative stress
- ——→ Biosynthetic pathway
- ⟹ Process activation
- e Control exerted by enhancement
- S Control exerted by suppression
- * Protoplast as conceived here evolved from cytoplasmic units released, after growth sphere envelope lysis, into buffered multiionic broth.

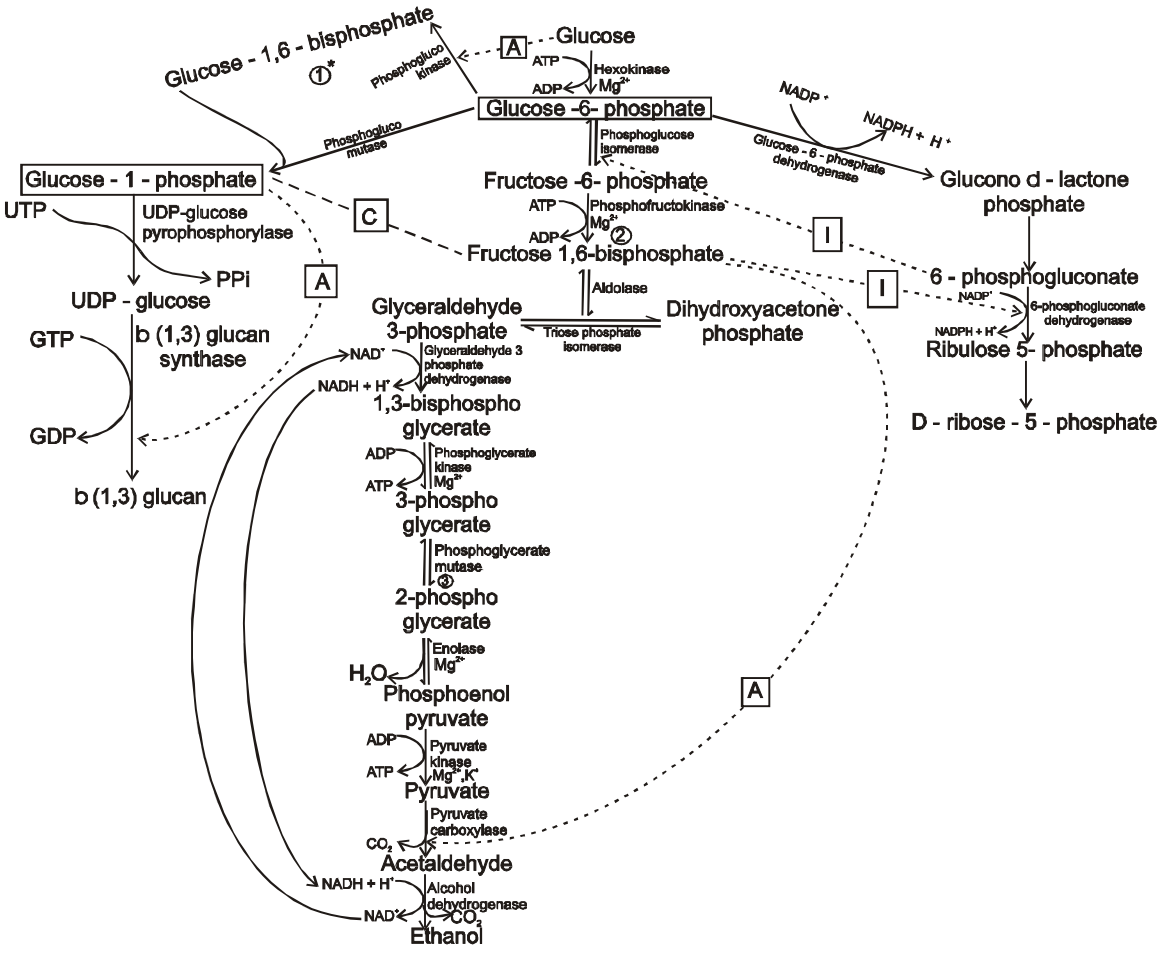

***Fig. 28*:** Scheme illustrating possible relationships in various pathways to glucose utilization in strain C13, IMI W5132B cultivated in multiionic buffered broth, pH 4.5 at 20^0C. Regulatory sites were indicated as follows, A = activation, I = inhibition, C = co-variation.
*Numbers indicated reaction steps where primers were required for execution. (1) Glucose - 1,6 - bisphosphate, (2) Fructose- 2,6-bisphosphate, (3) 2, 3-bisphosphoglycerate.

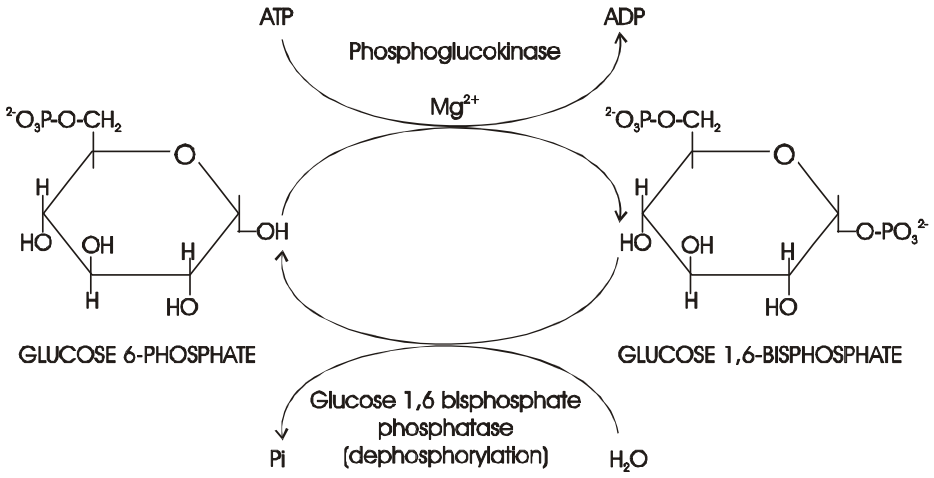

Fig. 29: Scheme illustrating the possible effect of phosphoglucokinase in generating primer for PGM activity in yeast cell wall biogenesis of strain C13, IMI 5132B cultivated in buffered multiionic broth at 20^0 C, pH 4.5 for 120h. Similar reaction occurs in glycolysis where fructose 2,6 bisphosphate acts as a primer for PFK (Van Schaftingen *et al.,* 1981; El-Maghrabi *et al.,* 1982; McGilvery, 1983; Harris, 1997).

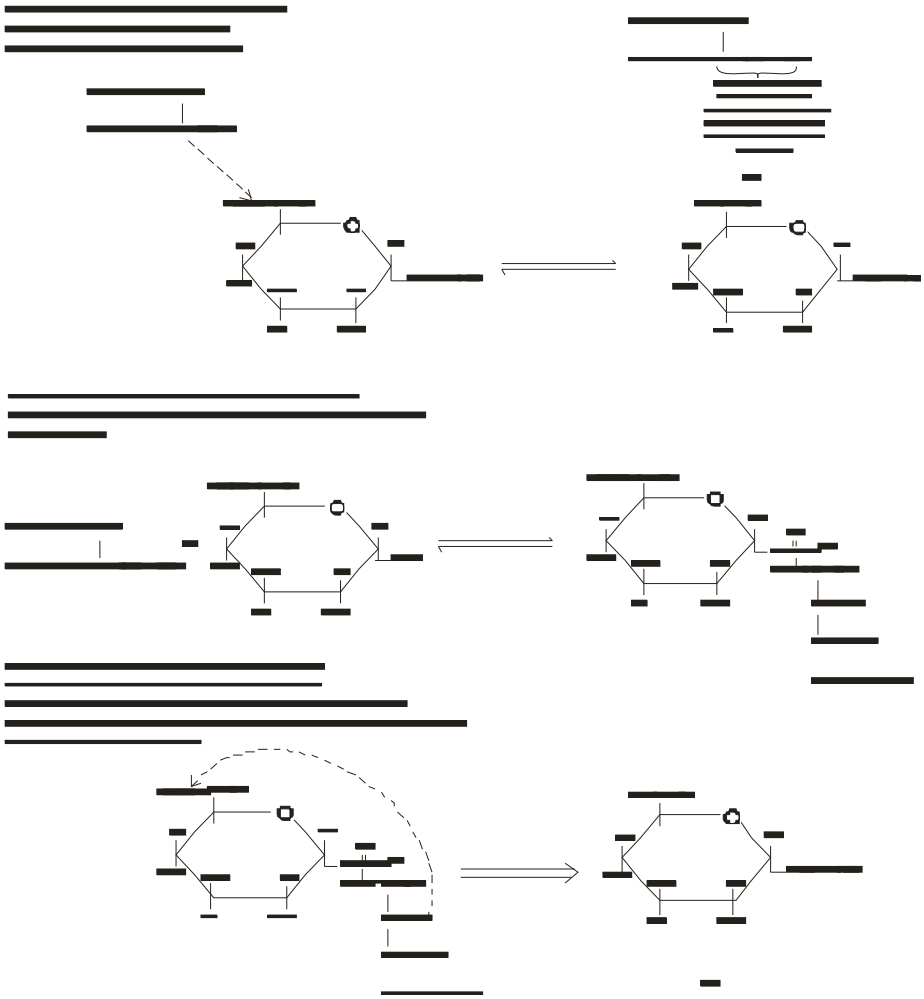

Fig. 30: Scheme illustrating the possible action of primer-induced phosphoglucomutase catalytic activity during the cultivation of strain C13, INI W5132B in multiionic buffered broth at 20°C, pH 4.5 for 120h. The catalytic effect of PGM

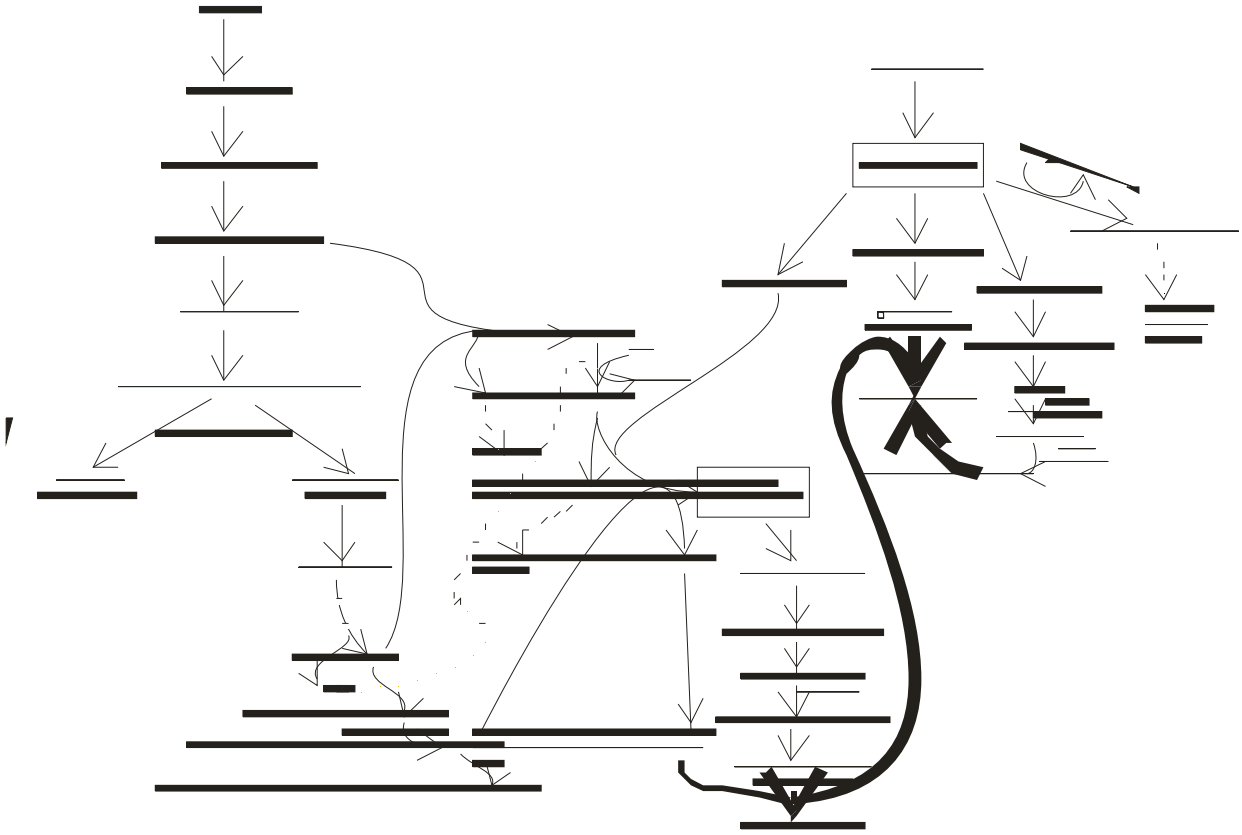

Fig 31: Scheme illustrating possible pathways used for carbon dissimilation and subsequent morphologies in tryptophan-sourced broth.

with UTP in the presence of UDP-glucose phosphotransferase would convert it to the sugar nucleotide and pyrophosphate kept low in the reaction system by the hydrolytic action of inorganic pyrophosphates. The free energies of reaction generated from both the hydrolytic action of the two enzymes would drive the process to the formation of the activated sugar (Gabriel and Van Lenten, 1981; McGilvery, 1983; Voet and Voet, 1995).

Up to the point of derivation of activated glucose reactions leading to formation of glycogen on the one hand and glucan on the other, would be the same. Thus, it was thought that similar phosphofructokinase: phosphoglucomutase coordinated regulation would be involved in the later, a process that differed only in the enzymatic conversion of UDP –glucose to β – 1, 3 glucan: biocatalyst GTP-glucan synthase (Cabib *et al.,* 1988), whereas ADP-glucose synthase converted same, to glycogen (Dietzler *et al.,* 1974, 1975). In the multiionic broth used in our studies, it was envisioned that a variety of interactions occurred which could affect not only the semi permeability characteristics of the plasma membrane, but, also its integrity. These, as noted by Jain (1972), could lead to the complete disruption of its functions. Thus the membrane needed protection against such destructive effects. Hence, it was thought, the cell wall origin. In cell wall biogenesis, the biopolymers are deposited external to the plasmamembrane. The cell wall also offers shape, stability of shape, maintenance of structural integrity of the cell and acts as filters, allowing molecules of moderate size to diffuse through it as well as acting as anchor for certain enzymes (Arnold, 1981; Suomalainen and Nurominen, 1973). It appeared, in our study, that in metabolic terms, energy generated at substrate level phosphorylation was preferentially harnessed for the construction of the structural polysaccharide, β - 1, 3 glucan, in a manner similar to *S. cerevisiae* cell wall biogenesis. Therefore, culpable cell wall biosynthetic reactions would be as shown below

Reaction			Enzyme
1. Glucose 1-phosphate +UTP	→	UDP - glucose + PPi	UDP- G pyrophosphorylase
2. PPi + H_20	→	2Pi	inorganic pyrophosphorylase
3. UDP – glucose	→	UDP + glucan	β - 1, 3 glucan synthase
4. UDP + GTP	→	UTP + GDP	nucleoside diphosphate kinase

Carbon dissimilation by most species of yeast is through the EMP pathway (Barnett 1976). Inderlied and Sypherd (1978) found that the yeast morphology of the dimorphic fungus *M. racemosus* catabolized most of the glucose in the medium of growth through the EMP pathway producing ethanol, CO_2 and glycerol, while 14-20% of the glucose was channeled through the PPP. Our contention on carbon metabolism by transformed yeast form of strain C13, IMI W5132B was that glucose carbon also substantially mobilized for cell wall biogenesis. In contrast to *M. racemosus,* which converted to multipolar budding yeast cells, the induced morphology of our dimorphic strain was terminal budding yeast cells, and these were capable of heterofermentative metabolism (*in press*).

Thus, the formation of terminal budding yeast cells in $(NH_4)_2SO_4$-sourced multiionic broth perceivably involved concerted effort by PPP, EMP pathway, and the mobilization of glucose carbon from G6P through G1P to the synthesis of β -1, 3 glucan in the protoplast of strain C13, IMI W5132B (Fig. 28). The occurrence of hyphal fragments and holoblastic conidia alongside the terminal budding yeast cells which predominated when tryptophan was the sole nitrogen source (*in press*) called for plausible explanation.

The possibility for the generation of superoxide anion during the cultivation of strain C13, IMI W5132B has been suggested. It was also noted that the anion was used in the catabolism of tryptophan in cells. Since it is the main rate determining factor in such cells (Hayaishi, 1978), that growth of strain C13, IMI W5132B occurred in tryptophan-sourced multiionic broth meant that superoxide anion was generated and sufficiently utilized to permit that level of growth (*in press*). It would also be extrapolated that metabolites arising from tryptophan breakdown induced hyphal fragments in addition to terminal budding yeast cells in contrast to $(NH_4)_2SO_4$-sourced multiionic broth, which induced only terminal budding yeast morphology from sporangiospores of strain C13, IMI W5132B.

Perhaps cleavage of the pyrrole ring by the superoxide-dependent tryptophan, 2, 3 dioxygenase provided substrate for kynurenine formamidase action and the kynurenine formed was hydroxylated in the presence

of a hydroxylase and reduced NADPH, presumably generated at the G6PD reaction of the PPP to give 3-hydroxy kynurenine. The alanine moiety, on being cleaved by a kynureninase, was made available for transamination reactions. The phenyl residue, 3-hydroxyanthranilate, could be opened up by another superoxide ion-activated enzyme, hydroxyanthranilate 3, 4- dioxygenase, thus providing the important substrate 2-Amino-3-carboxymucomic semialdehyde which could be quantitatively metabolized through the one pathway to nicotinate mononucleotide, and another, involving a number of secondary products including complex reaction which generate NH_4^+, glutamate and acetoacetyl CoA (McGilvery, 1983; Voet and Voet, 1995; Coomes, 1997). The possible pathways used for carbon dissimilation and subsequent morphologies in tryptophan-sourced broth were shown in fig. 31.

We proposed that in our system, a possibility existed for NH_3 derived from this process to be used for glutamine synthesis (in the presence of glutamine synthetase) and subsequently to amidify glucose 6-phosphate, catalyzed by glutaminase, thus yielding N-acetyl glucosamine 6–P, which became activated to UDP-glucosamine N-acetyl CoA. Further transformation would generate the substrate UDP-N-acetyl glucosamine, which was then converted by chitin synthetase in a one- step reaction to N-acetyl glucosamine, the microfibrillar structural material for hyphal morphology. Studies have shown that chitin polymers could be overlaid with amorphous material originating from β– 1, 3 glucan as in *M. rouxii* (Bartnicki-Garcia, 1968), α- 1, 3 glucan as in *P. brasiliensis* (Carbonell *et al.,* 1970), or α - and β- 1, 3 glucan as in *Crytococcus* and *Schizosaccharomyces* species (Bacon *et al.,* 1968). But chitin layer constituted the greater proportion of hyphal wall in *M. rouxii* (Dow and Rubery, 1977) and *C. albicans* (Braun and Calderone, 1978) whereas the site of first deposition was at the growing hyphal tip of fungi (Bartnicki- Garcia and Lippman, 1977; Bartnicki-Garcia, 1987; Braun and Calderone, 1978). Hyphal wall construction by strain C13, IMI W5132B in tryptophan-sourced buffered multiionic broth could be similar to that of other fungal species. Fig. 31 showed a scheme leading to hyphal and yeast wall biogenesis by strain C13, IMI W5132B in this medium.

When the aromatic compound tyrosine was the sole nitrogen source, terminal budding yeast cell was the sole morphology induced. The secondary products of tyrosine catabolism include alanine (which could be used for transmutation reactions), fumarate and acetoacetate (Coomes, 1997). Facultative microorganisms like *E. coli,* contain fumarate reductase, which provides a method for succinate formation under anaerobic conditions (Moat, 1979). Since strain C13, IMI W5132B proved to be a facultative microorganism, it probably contained fumarate reductase (as distinct from aerobically-dependent succinate dehydrogenase), which enabled it to utilize fumarate condensed from acetoacetate derived from tyrosine catabolism in anaerobic conditions, a prerequisite for the yeast morphology. Reported elsewhere is the chromatographic detection of succinate in media seeded with primed yeast cells of strain C13, IMI W5132B.

The induction of hyphal/thallic growth from sporangiospores of strain C12, IMI W5132A in glucose – substrate multiionic broth (Omoifo, 1997) contrasted with the yeast morphology transformed from sporangiospore of strain C13, IMI W5132B in similar medium (Omoifo, 1996b; 1997). This meant that there was fundamental difference in the utilization of similar substrate by the two strains. This difference could be at the enzymic level. Like strain C13, IMI W5132B, protoplasts were observed in the transformation process of strain C12, IMI W5132A. This indicated that the morphological changes that occurred during the first half of the transformation process were the same for the two strains. Since the elemental composition of the growth media was the same for both strains, we took the liberty to assume that RO1s were also generated during the growth of strain C12, IMI W5132A. These would be disproportionated by antioxidants in other to prevent destruction of cellular components. Such reactions made molecular oxygen available in the broth. Such could impose the 'oxygen effect' on biochemical reactions. By the scheme shown in fig. 26, the PGM, PFK and pyruvate kinase activities would be greatly affected.

A fall out of this was the Pasteur effect that allowed less substrate utilization for the greater amount of energy produced through the TCA and oxidative phosphorylation; it would also permit cell wall biogenesis attuned to the high ATP-generating system. Vegetative wall was the presumable result. Since it was the possible conversion of G6P to G1P that was prevented, it was thought that the generated RO1s or the disproportionation products inhibited the phosphogluckinase and phosphoglucomutase activities. Induction of enzymes for vegetative wall formation would then give orientation to growth. Protoplasts have been shown to exhibit polarized growth (Carbonell and Yegres, 1973; De Vries and Wessels, 1982), as vegetative wall was deposited external to it (Carbonell *et al.,* 1973).

The simultaneous induction of club-shaped terminal budding yeast cells and thallic structures from sporangiospores of strain C12, IMI W5132A inoculated into galactose –substrated multiionic broth

(Omoifo, 1997) contrasted with the ellipsoidal terminal budding yeast, thallic and additionally, multipolar budding yeast-like morphology induced from sporangiospores of strain C13, IMI W5132B cultivated in similar medium (*in press*). Although the adaptive history of an organism plays a role in its metabolism, as Season *et al.* (1973) showed that *S. carlsbergensis* strain 74 primed with galactose produced acid in galatose- medium but not so when priming was done with glucose, morphological differences in our two strains were considered specific to each strain. Since terminal budding yeast- and hyphal morphologies were expressed by either strain, the physiological processes leading to such expression might be similar.

Consider the fate of galactose in the protoplast, after proton-substrate symport (Season *et al,* 1977), in the presence of inducible galactose permease, then phosphorylative effect of galactokinase would yield galactose 1-phosphate epimerizable to glucose 1- phosphate. This ester, when activated became substrate and effectors for glucan biogenesis in the presence of the rate-limiting enzyme, glucan synthase, as did glycogen synthase for glycogen biogenesis (Dietzler *et al.,* 1974, 1975). Glucan synthesis, coordinated with substrate level phosphorylation, would give rise to the yeast morphology.

As growth spheres did not lyse at the same time (Omoifo, 1996b), indicating that there were differences in the rate of isotropic growth of sporangiospores, it was possible that some pre-lyses spheres, after cessation of isotropic growth, were subjected to localized growth thus displaying wall synthesizing activity that resulted in germ tube formation (Bartnicki-Garcia, 1968; Bartnicki-Garcia *et al.,* 1968; Bartnicki-Garcia and Lippman, 1977) apparently as response to the oxygen effect (Fig. 26). Such could result in the formation of holothallic conidia (Fig. 17, 32). Several germ tubes per growth sphere could be so formed, a development that occurred in *M. rouxii* only during aerobic growth (Bartnicki-Garcia and Lippman, 1977). Bartnicki-Garcia and Lippman (1977) used autoradiographic means to show that germ tube emerged from the vegetative wall synthesized underneath the spore wall, a predominantly glucan polysaccharide (Bartnicki-Garcia and Reyes, 1964). But wall materials of the germ tube consisted of amino polysaccharide, chitin (N-acetylglucosamine) and chitosan (glucosamime) (Bartnicki-Garcia and Lippman, 1977), transformed from the glycolytic intermediate, glucose 6- phosphate (Gabriel and Van Lenten, 1978; Zubay *et al*., 1995). It was our considered opinion that the physiological coupling of chitin and chitosan biosynthesis to oxidative phosphorylation gave rise to mould morphology within the galactose -, or glucose-substrated multiionic broth. The development within the broth, of coenocytic non-columellate sporophores apically bearing multispored sporangia originated from branched septate mycelia of strain C12, IMI W5132A, was conspicuous when glucose-substrate multiionic broth was incorporated with nicotinic acid. Although the asexual reproductive apparatus was atrophied, they were numerous. Such was also observed when riboflavin (precursor for FAD^+, coenzyme for oxidative phosphorylation) was incorporated into the growth medium (Omoifo, 1997).

The fact that yeast cells were not induced from sporangiospores of strain C12, IMI W5132A in glucose-substrate multiionic broth could therefore be attributed to enzyme sensitivity to molecular oxygen insult. Apart from phosphoglucokinase and phosphoglucomutase mentioned above, other enzymes that could be inhibited include phosphofructokinase and pyruvate decarboxylase (Oura, 1974b). In his earlier study, Oura (1974a) found that increasing levels of molecular oxygen content progressively weakened anaerobic fermentative metabolism until a low point where aerobic oxidative metabolism took over. A tension of 26liter gas /hr/litre culture which contained 25-30% oxygen was necessary to induce a completely oxidative metabolism. Assuming similar effect in our glucose-substrate multiionic broth, then, facultative strain C12, IMI W5132A would adopt the oxidative phosphorylation coupled to chitin and chitosan biosynthesis.

When strain C12, IMI W5132A utilized maltose for growth, induced were units of the transient morphology, hypha fragments, and terminal budding yeast cells, which were predominant (Omoifo, 1997). Although the hypha fragments were scanty, their occurrence required similar consideration as discussed for other sugars. Intracellular maltose undergo phosphorolosis at the non-reducing end giving rise to glucose 1-phosphate, a ready substrate for glucan biosynthesis, while the other glucose unit phosphorylated at the C6-OH catalyzed by hexokinase, formatted the glycolytic process. A consolidated action between the EMP process and glucan biogenesis would thus yield the yeast morphology. Beside this, transmutation reactions involving acetomido group coupled with 'oxygen effect' – driven oxidative metabolism could transform glucose 6-phosphate to amino polysaccharides, probably in an inconsistent faction, to give hyphal fragments.

When malic acid was the substrate for the growth of strain C12, IMI W5132A, the morphologies induced were similar to that discussed for maltose (Omoifo 1997), an indication that with both carbon sources physiological processes for cell wall construction were similar. But biomass obtained with maltose,

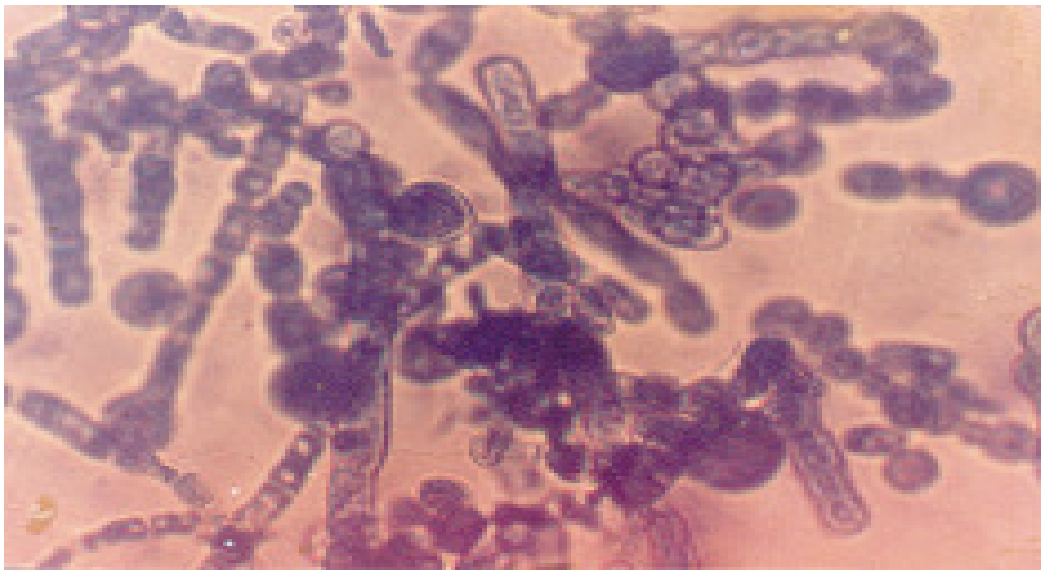

Fig. 32: Holothallic conidia induced from sporangiospores of strain C12, IMI W5132A cultivated in glucose-substrated multiionic broth for 3 days, pH 3.5 at 20°C, ambient.

as substrate was 4.3- folds that of the malate-medium (Omoifo, 1997). This showed that growth was more vigorous in the maltose-substrate multiionic broth.

Biochemical considerations indicated that gluconeogenic process, with malate as starting material, provided glycolytic intermediates, which could be used in other transformative processes. In the trypanosome, *Trypanosoma cruzi,* induction of allosteric NADP-linked malic enzyme was the first reaction step in the gluconeogenic process where malate was decarboxylated to pyruvate, a reaction inhibited by oxaloacetate (Cazzulo *et al.,* 1977). The decarboxylation step occurred in the cytosol and pyruvate formed was converted to phosphoenolpyruvate (PEP) in the presence of PEP carboxykinase in the mitochondrion matrix. PEP therefore shuttled out of the mitochondrion and into the cytosol where it would continue the carbon structure build up using the glycolytic enzymes in the reverse order, except at the PFK reaction step which was replaced by the inducible fructose 1,6 bisphosphate (Shrago *et al.,* 1963; Yudkin and Offord, 1976). Once achieved, glucose 1-P could be partitioned into the various metabolic pathways as shown in fig. 28. Fig. 33 illustrated the possible pathways for carbon utilization by strain C12, IMI W5132A.

The malate-gluconeogenic process apparently involved more reaction steps and energy expenditure to reach G6P than the maltose conversion to the same ester. The data of Omoifo (1997) showed that in non-buffered multiionic broth, maltose substrate yielded 4.3- fold more biomass of strain C12, IMI W5132A than malate substrate while it was 20.3-fold advantage when the broths were buffered. Thus, this confirmed the relative advantage in using maltose substrate in the sporangiospore-yeast transformation of strain C12, IMI W5132A.

viii. Possible mechanism for utilization of second messengers

The data of Omoifo (1997) showed that biomass of strain C12, IMI W5132A in inositol-incorporated broth was 1.83 times higher than that in the non-vitamin rich medium. Furthermore, two distinct morphologies were induced in the former: mycelia and yeast (terminal budding). However, the filamentous form was predominant. We have suggested above that glucan biogenesis was coupled to fermentative metabolism for yeast form development while similar effect occurred with chitin and chitosan biogenesis and oxidative phosphorylation for filamentous growth.

This morphogenesis – physiology parallelism agreed with that of Stewart and Rogers (1978). Since it was the incorporation of inositol into the glucose – substrate multiionic broth that induced the higher biomass, as well as yeast morphology, then its effect required plausible explanation.

Reber *et al.* (1977) demonstrated that exogenously supplied inositol was actively transported through the membranes of *Klebsiella aerogenes* in a proton - inositol coupled electrogenic relationship. In the cytosol, inositol participated in the phosphatidylinositol cycle, a physiological response that generated second messengers, diacylglycerol (DAG) and inositol 1, 4,5 trisphosphate ($1P_3$), which participate in signal transduction for the activation of cellular functions and proliferation (Turner and Kuo, 1985; Kikkawa *et al.,* 1985; Anderson and Salomon, 1985; Berridge, 1987).

The protoplast, as conceived here evolved as a multi-dimensional structure capable of self-reorganization, a consequence of coordinated control of interacting species with specific molecular structures. Thus cytoplasmic granules, after growth sphere envelope lyses, became exposed in the buffered multiionic broth; a possibility existed for spontaneous formation of a bilipid layer which then became thermodynamically stable, giving rise to lipid- protein interactions and exhibiting versatility influenced by slight changes in parameters such as ionic strength, pH, concentration of divalent cations and temp (Jain, 1972; Trouble and Eibl, 1975). In our phosphate- rich broth, it was thought, charged phosphate ester groups became incorporated into the membrane lipid in order to further attract water. The lipid component or phosphatidic acid consisted of fatty acids, i.e., esterified glycerol on two carbon atoms, and a phosphate group on the third.

With inositol available in the cytosol, a reaction could occur between the hexitol and phosphatidic acid driven by cytidine triphosphate (CTP) thus forming phosphatidyl inositol (Fig. 34). In an acid environment, this compound would be attracted to the membrane bound phosphatidyl serine. Membranes-associated kinases would phosphorylate the inositol head group yielding phosphatidylinositol 4 - phosphate (P1P). Further phosphorylation, at position '5' would yield phosphatidylinositol 4, 5-bisphosphate ($P1P_2$). Signal transduction involved hydrolysis of the $P1P_2$ by a phosphatidylinositol (P1) - specific phospholipace C to generate second messengers including 1, 2- diacylglycerol (DAG) and inositol 1,4, 5- trisphosphate ($1P_3$). The water soluble$1P_3$ then diffused from the membrane inner - leaflet into the cytosol thus binding to volutin or endoplasmic reticulum - originated Ca^{2+} store, whereby it stimulated the release of Ca^{2+} ions into

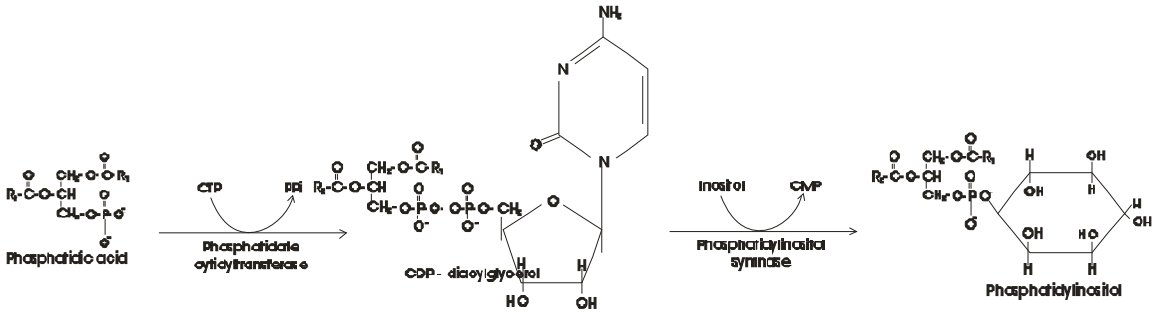

Fig. 34: Possible biochemical reactions leading to the formation of phosphatidylinositol in the cytosol of protoplast of strain C12, IMI W5132A cultivated in glucose - substrated inositol - incorporated multiionic broth at 20°C, pH 3.5 ambient.

the cytosol. The Ca^{2+} ions in turn would migrate through the cytosol to activate the protein kinase system, important in cellular metabolism (Anderson and Salomon, 1985; Berridge, 1987; Kikkawa *et al.,* 1980; Litwack and Schmidt, 1997; Voet and Voet, 1995). Protein kinase C, which has absolute requirement for Ca^{2+}, is present in all living tissues (Kikkawa *et al.,* 1985). Aquino – Pinero and Rodriguez –del- Valle (1997) detected the activity of PKC in both mycelia and yeast forms of the dimorphic fungus S*porothrix schenchia* by use of the phorbol ester, 12 - myristate –13 – acetate phorbol. Therefore, it was suggested that PKC was also present in strain C12, IMI W5132A. Litwack and Schmidt (1997) stated that the kinase exhibit heterogeneity. This appeared to be an indication of multiple functionality of the enzyme.

Alexander G. Newton (1995) reviewed the protein kinase Cs and separated them into three different groups (i) conventional, (ii) novel and (iii) atypical, each being equiped with a catalytic and regulatory domain which rendered the enzyme competent for its functional role, as well aligning substrate for effectivity. Accordingly, the conventional PKC was equiped with a diacyl-binding site followed by an encompassing acid phosphatilylserine recognition site, and Ca^{2+} binding site so important in its regulatory functions. However, the novel PKC subspecies at their recognition sites lacked the functional groups, which would mediate Ca^{2+} binding, even though the structural folds similar to the conventional type were maintained. The atypical type on the other hand, lacked both sites. Litwack and Schmidt (1997) noted that the PKC subspecies had two Zn^{2+} fingers. These could well be the novel- PKC subspecies. The experiments of Aqiuino- Pinero and Rodriguez-de Valle (1997) have emphasized this: while yeast and mycelia forms of dimorphic *S. schenckii* contained gamma and zeta isoforms of PKC, the beta isoform was detected only in the yeast form. It was pertinent to point out that *S. schenckii* exists as spherical to ovoid budding yeast cells at elevated temp, but mould form at room temp (Jawetz *et al*., 1980).

Assuming the occurrence of the novel PKC subspecies in strain C12, IMI W5132A growing in the buffered multiionic broth, pH 3.5, that lacked Ca^{2+} but was enriched with Zn^{2+} ions, then the internal signal transducing system could have been activated. This implied that Zn^{2+} ion, rather than Ca^{2+} ion, played the important role as a regulator of cell growth in strain C12, IMI W5132A physiology. That exogenously applied zinc reversed EDTA growth inhibition and induced formation of multipolar budding yeast-like cells of *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962b), or progressively converted filamentous growth of *C. albicans* to the yeast form, with increasing concentration until the optimum level when growth was completely in terminal budding yeast form (Yamaguchi, 1975), lent support to this thesis.

Since we assumed Zn^{2+} ion activation of novel PKC isoform, a higher level of the cation would cause the activated PKC to migrate, as Ca^{2+} does in the conventional PKC, to membrane where they form ligand-receptor relationship with membrane bound negatively charged phospholipid. Such ligand binding has been found to be selective for the lipid head group, phosphatidylserine, which is indispensable for PKC activation in mammals (Kikkawa *et al.,* 1985). Generally, phosphatidylserine showed greater cooperativity than phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, which gave an opposite effect to the former (Newton, 1995). Kerridge *et al.* (1976) found that protoplasmic membranes of exponentially growing yeast cells of *C. albicans* contained the aforementioned species of phospholipids; phosphatidylserine (including phosphatidylinositol) amounted to 11±20% in yeast membrane but 0% in the mycelia form.

Assuming this phosphatidylserine composition of the membrane of strain C12, IMI W5132A, and also the non–polar DAG second messenger, which remained at the plane of the membrane after formation, would heighten the selectivity and affinity of its substrates (Newton, 1995). Thence, the Zn^{2+}- stimulated novel PKC translocated from the cytosol would be synergistically activated in the presence of phosphatidylserine by phosphorylating it at specific protein. This in turn modulated other activities in cellular metabolism (Litwack and Schmidt, 1997; Voet and Voet, 1995).

This conjectural theme was derived from the fact that in platelets, model for many works on signal transducing possibilities in cells, synergistic interactions occurred between DAG, the negatively charged membrane-bound phosphatidylserine and intracellular calmodulin- mediated Ca^{2+} (Ca^{2+} release from Ca^{2+} store induced by IP_3) with the PKC family of enzymes (Kikkawa *et al*., 1985). From the review of Berridge (1987), three clear pathways for the action of activated PKC could be deduced: (i) mitogenic stimulation, (ii) Na^{+}-H^{+} exchange and pH regulation, and (iii) ionic modulation. Apart from the effect on nPKC itself, it was likely that ionic movement played other roles, which could influence the potentiality of the growing cell. The study of Paveto *et al.* (1975) showed that Mn^{2+} was required for the activity of adenylate cyclase, a membrane bound enzyme, which stimulated the formation cyclic $3^{/}$, $5^{/}$ -adenosine monophosphate (cAMP). The cAMP in turn induced yeast-like cells of *M. rouxii*. These workers found that the divalent cation, Mg^{2+}, could only replace 2% of the Mn^{2+} activity. On the other hand, Mg^{2+} - ATP complex, a core

ligand in the cell's metabolic status (Blair, 1970), on elevated conversion to cAMP, a second messenger (Sutherland, 1972; Abeles *et al.,* 1992; Cory, 1997) by Mn^{2+} requiring adenylate cyclase could chart physiological direction for the cell's metabolism. Larsen and Sypherd (1974) showed that exogenously supplied cAMP induced the transformation of sporangiospores to multipolar budding yeast-like morphology of *M. rouxii*, a finding confirmed by Paveto *et al.* (1975). This effect indicated that a mechanism for block yeast-like-enzyme expression was considered to be in operation.

Elevation of intracellular concentration of cAMP enabled *E. coli* to overcome both transient and permanent catabolic repression by glucose (Pastan and Pearlman, 1970; Pearlman *et al.,* 1970). In *E. coli*, cAMP binds specifically to a dimeric protein, cyclic AMP receptor protein (CRP), forming a complex, which allowed the former to undergo a large conformational change thus enhancing its affinity for DNA (Pastan and Adhya, 1976). A three- fold rise in cAMP level saturated the cAMP-CRP complex making it bind tightly at specific DNA promoters (Busby and Buc, 1987). The positive binding of the complex to the target promoter would activate RNA polymerase thereby turning on the transcription machinery (Pastan and Adhya, 1976; Busby and Buc, 1987; Voet and Voet 1995).

The mode of signal transduction in eukaryotes appeared to be different. Lailli and Sussone- Corsi (1994) explained that cAMP would bind with protein kinase A (PKA) at its regulatory unit, which then released its active catalytic subunit. Thus activated, the catalytic subunit translocated to the promoter region and hence activated transcription of inducible genes. It was also noted that PKC, with the ability to stimulate mitogenesis and growth, could bind at the same target promoter as PKA (Litwack and Schmidt, 1997).

Although no specific experiments have been conducted on the intracellular signal transducing capabilities of strain C12, IMI W5132A, theoretical analysis seemed to suggest that exogenously applied inositol in glucose-substrate multiionic broth triggered the generation of second messengers including $1P_3$, DAG and cAMP and their intracellular signaling possibilities which thereafter activated specific sequences in the promoter domain thus, stimulating DNA-dependent RNA polymerase. This in turn activated the transcription of inducible genes that gave rise to block enzymes. These probably included phosphoglucomutase, phosphoglucokinase, glucan synthase and, phosphofructokinase, fructose 2, 6 bisphosphate, phosphoglyceromutase, pyruvate kinase and alcohol dehydrogenase as well as phosphatidylinositol synthase, kinases, phospholipase, inositol phosphatases and CTP-phosphatidate cytidyl transferase. Fig.35 showed a proposed scheme for second messenger generation and signal transduction that led to the evolvement of terminal budding yeast morphology from protoplasts. In such evolved yeast, intracellular reactions catalyzed by phosphatases consequently converted $1P_3$ to inositol; thereafter, it was available for further action by phosphatidyl synthase. Since this was the starting point, a full cycle known as the phosphatidylinositol (P1) cycle could thus be accomplished. Combining with CDP-phosphatidic acid and DAG could recycle this, which in the presence of DAG-kinase often accompany the P1 response (Turner and Kuo, 1985; Voet and Voet, 1995). The presence of externally supplied inositol, it was thought, made continuation of this cycle inevitable.

Since sporangiospores of strain C12, IMI W5132A converted to sole mycelia morphology in glucose substrated multiionic broth, it probably meant that the P1 response was not operative in such morphology induced, or lacked specific proteins for the 'yeasting process', in this medium. The substrate for synthesis of inositol was the glycolytic intermediated, G6-P, which was converted to G1-P, followed by intramolecular oxidation-reduction reactions and subsequent removal of the phosphate group (McGilvery, 1983). It seemed this was the transformative process that was blocked during the growth of strain C12, IMI W5132A in glucose-substrated multiionic broth without growth factor incorporation. In the event of this, physiological response that led to only hyphal growth was permitted.

Fig. 36 indicated that the initial biochemical reactions involved in the hexitol biosynthesis shared the same intermediates with biosynthesis of glucan, the yeast wall structural material, with G-1-P being the cross over point. Therefore it was suggested that the lack of synthesis of G-1-P by strain C12, IMI W5132A in glucose- substrated multiionic broth, that is, without inositol incorporation, affected both β- 1, 3 glucan and phosphatidylinositol generation and turnover and, hence, yeast formation. Blockage of this biochemical process could be at the level of phosphoglucomutase (Fig. 36), its activity thought negatively affected by the oxygen effect (Fig. 26). Instructive here was the chromatographic detection of the same types of DNA dependent RNA polymerase in both yeast and mycelia forms of *M. rouxii* by Young and Whiteley (1975). Therefore the divergent morphological expressions of this organism might not be attributed to differences in genomic transcriptional ability.

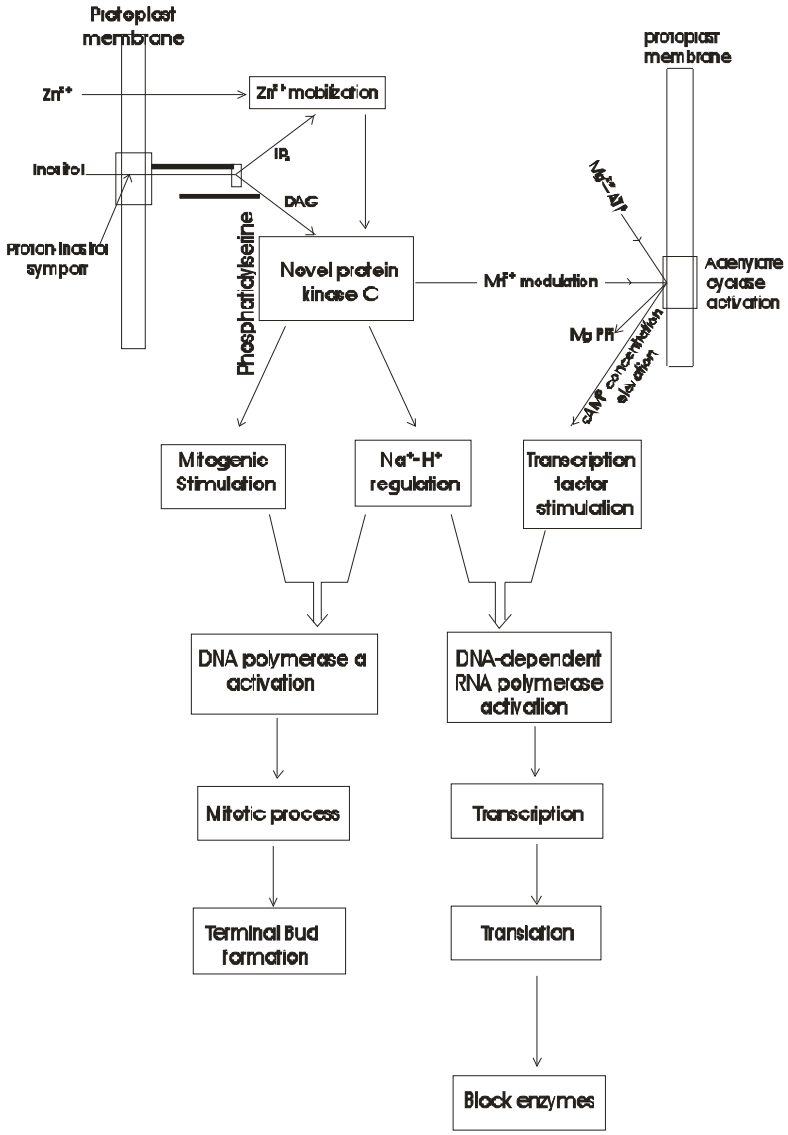

Fig. 35: Scheme illustrating possible role of second messengers and ionic species in signal transduction for full physiological response leading to yeast cell induction during the growth of strain C12, IMI W5132A in inositol - incorporated glucose - substrated multiionic broth at 20°C, pH 3.5, ambient. Note: The medium was not buffered and both yeast cells and filaments were simultaneously induced from sporangiospores.

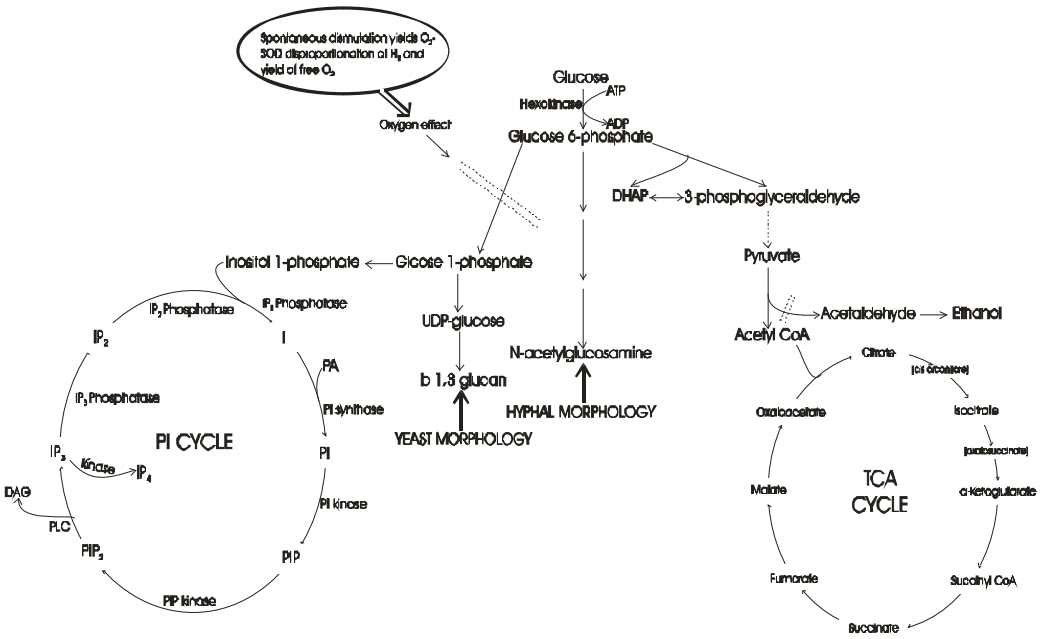

Fig. 36: Scheme illustrating possible pathways for glucose utilization, oxygen effect and mycelia induction during the cultivation of strain C12, IMI W5132A in multiionic broth at 20°C pH 3.5 (Omoifo, 1997). The double hatched lines indicate the possible reaction steps blocked thus giving prominence to the alternate physiology and form. Exogenous supply of inositol probably permitted the enhancement of the PI cycle thereby generating second messengers which executed signal transduction for the terminal budding yeast morphology.

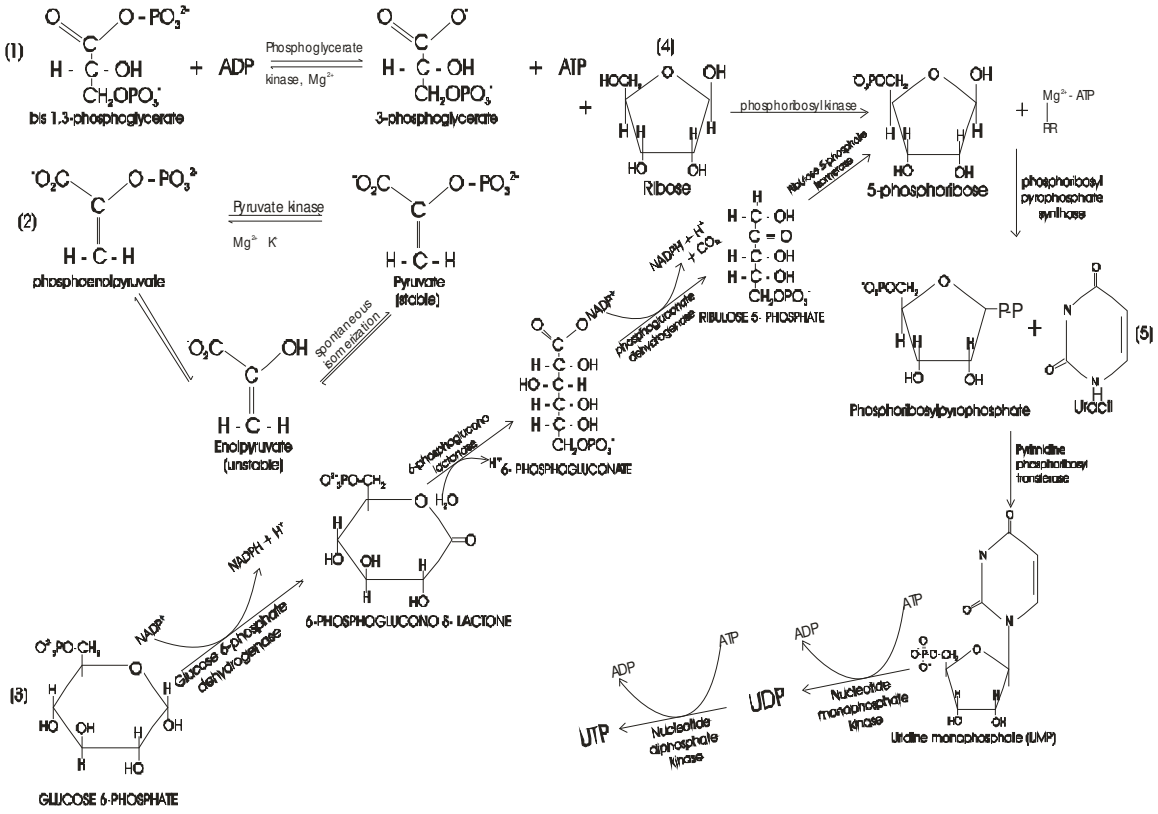

Fig. 37: Scheme illustrating possible salvage route for the synthesis of UMP during the cultivation of strain C12, IMI W5132A in glucose - substrated, uracil - incorporated multiionic broth at 20°C, pH 3.5 ambient. Numbers indicated the source of compound: (1) and (2) = glycolysis, (3) = exogenous supply of glucose, (4) = pentose phosphate pathway, (5) = exogenous supply.

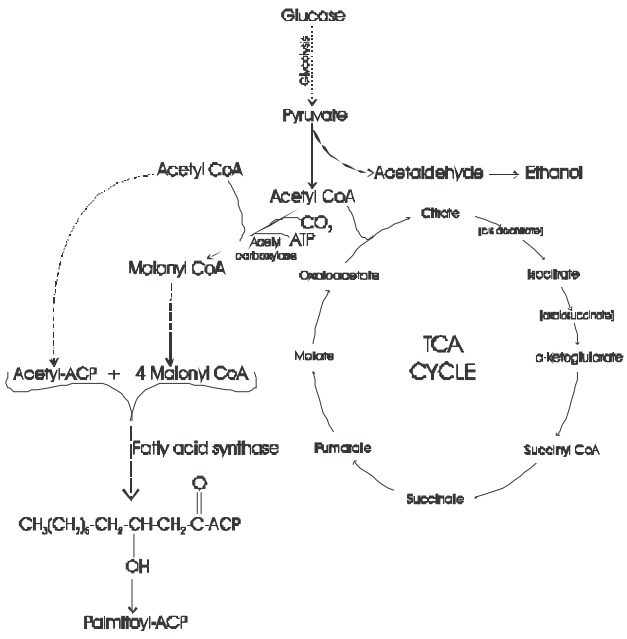

***Fig. 39 (ii)*:** Acetyl carboxylase multilenzyme complex

Fig. 39i: Condensation reactions starting with acetyl Co A lead to the synthesis of fatty acids

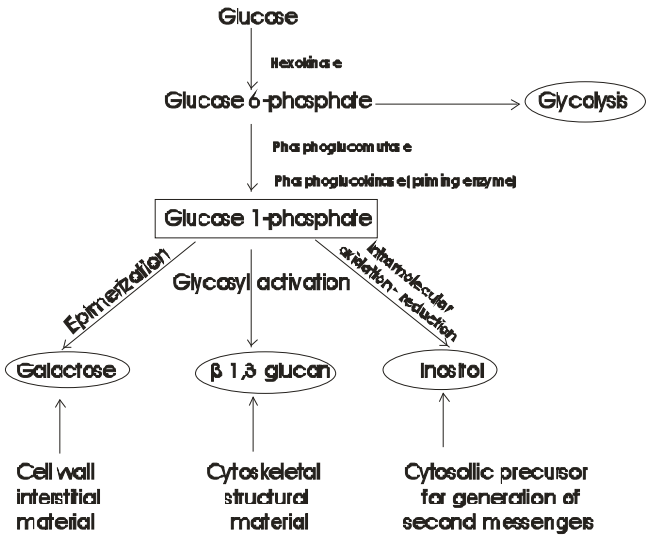

Fig. 40: Scheme showing glucose - 1 - phosphate as cross over point in the branch pathways for the synthesis of galactose, glucan and inositol. It was suggested that ambient conditions favoured the establishment of these physiological processes during the cultivation of strain C12, IMI W5132A in multiionic broths in which terminal budding yeast cells were induced from sporangiospores.

ix Possible mechanism for metabolic route switching and phosphatidylinositol response

The biomass of strain C12, IMI W5132A in uracil-incorporated glucose-substrated multiionic broth was comparable with that in similar medium but without growth factor incorporation (Omoifo, 1997). A remarkable difference between the two cultures was seen in the morphological expression. Whereas form of growth in the control broth was submerged filamentation, it was mainly yeast cells in uracil-incorporated broth. Also noted was the extent of filamentation when either inositol or uracil was incorporated into the growth medium. Whereas it was fully extensive with inositol, hyphal fragments did not develop into colony, patchment or clump in uracil-fed medium. Yet, yeast cells were more preponderant with the latter.

Uracil is absolutely required in the formation of nucleotides. It also plays critical roles in cellular metabolism. For instance, it is specific to RNA. Therefore, in proliferating cells, transcription of genetic information into messenger RNA represented veritable platform for uracil utilization. Uridine nucleotide could be synthesized through several salvage routes to give UMP that could be converted to ATP-driven UTP in the presence of nucleotide diphosphokinase (Abeles *et al.,* 1992; McGilvery, 1983; Cory, 1997). This was illustrated in fig. 37. UTP would serve as the direct precursor of CTP but under regulatory control so as to achieve a balance between the two nucleotides. On the other hand, TTP, a nucleotide specific to DNA and of absolute requirement during the *S*-phase of growth could be synthesized from CTP or UTP, using serine as the supplier of CH_3 group. These nucleotides function as energy carriers, regulatory molecules and as precursors to coenzymes and nucleic acids. Possibility for the generation of nucleotides, through salvage routes, was demonstrated by Simon *et al* (1990), who prepared UTP using the glycolytic intermediate phosphoglyceric acid as the phosphoryl donor, in the presence of the enzymes phosphoglycerate mutase, pyruvate kinase and adenylate kinase with $MgCl_2.6H_20$ as cofactor in an *in vitro* system that also incorporated the Na-salts of UMP and ATP. A high yield, 92%, of the nucleotide was obtained. Assuming the occurrence of such biochemical process in our glucose-substrated multiionic broth into which sporangiospores of strain C12, IMI W5132A were inoculated, then the high level supply of uracil could ensure that UTP supply was inexhaustive.

Glucose 1-P combines with MgUTP- complex in reaction catalyzed by uridine diphosphate glucose (UDG) and Mgpyrophosphate (MgPPi). This reaction occurred sequentially in compulsorily ordered binding process (Abeles *et al.,* 1992). The MgPPi formed would be further hydrolyzed by inorganic pyrophosphatase thereby keeping the pyrophosphate concentration low, and hence, in terms of energetics, favored UDP-glucose formation and irreversibility of reaction, fig. 38. Such high- energy status afforded by the ligated diphosphate ester would increase the reactivity of the glycosyl group, UDP-glucose, and act as donor for many biosynthetic reactions (McGilvery, 1983; Voet and Voet, 1995; Harris, 1997). Thus it was suggested that in the system used for the growth of strain C12, IMI W5132A, which had enhanced uracil supply, the experimentally determined changing intracellular pH (monitored in the bulk medium) (Omoifo, 1996b) in a phosphate-citrate buffered system and the accompanying intracellular conditions became appropriate for such biosynthetic reactions involving β 1, 3-glucan synthase (Fig. 33), the enzyme for construction of the yeast cytoskeletal structure. We presumed that this biosynthetic process acted in a coordinated manner with the P1 response that resulted in the activation of the protoplast transcript machinery. This possibly induced enzymes that catalyzed metabolic and cytoskeletal microfibrillar structures that gave the yeast morphology.

As stated above, inositol and phosphatidic acid could be involved in the P1 response. Since neither of these growth substances was exogenously supplied to the medium of growth, their involvement in the microorganism's metabolism could only be derived during the growth process. Perhaps the glycolytic intermediate, DHAP, provided precursors for glyceryl group. We assumed that in such transforming unit, there would be evolved full complements of intracellular organelles. Pyruvate, also a glycolytic intermediate, traversed the mitochondria membrane where enzymatic activities led to its multienzyme oxidative decarboxylation to Acetyl Co A (Fig. 39i). It combined with oxaloacetate to form citrate in which form it shuttled to the cytosol where the acetyl group would be given up for subsequent reactions. Acetyl Co A in turn combined with $C0_3$ in reactions involving a protein complex, including biotin carboxylase, biotin carboxylase carrier protein and carboxyl transferase to yield malonyl Co A (Fig. 39ii). Thereafter malonyl CoA and acetyl CoA would form co substrates for the synthesis of fatty acyl groups. A multifunctional enzyme, fatty acid synthase, would catalyze the seven reaction steps leading to the formation of palmitate in the first instance. Fatty acyl groups react with glycolysis – derived glycerol phosphate at carbon 1 (saturated) and carbon 2 (unsaturated) to yield phosphatidic acid (Voet and Voet, 1995). This process was diagrammed in fig. 39iii.

We assumed that the P1 cycle (Fig. 36) occurred during the growth of strain C12, IMI W5132A in uracil– incorporated glucose-substrate multiionic broth. Occurrence of the hexitol in the cycle also required a plausible explanation since it was not exogenously supplied. With reference to *P. brasiliensis* the induced level of PGM in the yeast form was higher than the base level in the mycelia form (Kanetsuna and Carbonell, 1966). Since galactose was a probable end- product of the chain of reactions involving phosphoglucomutase–catalyzed step and subsequent epimerization of intermediates, microorganisms that utilized this Leloir pathway would at a stage contain glucose 1-phosphate. Analyses had shown that galactose was a component of cell walls of mycelia and yeast forms of *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962d; Dow and Rubery, 1977; Ruiz-Heirera, 1985) and mycelia form and, to a lesser extent, yeast form of *B. dermatitidis* (Kanetsuna and Carbonell, 1971), which were cultivated in glucose- substrate media. Glucose 1-P, it appeared was the cross over point for many biosynthetic pathways including Leloir pathway, glucan biosynthetic pathway and inositol oxido-reductive pathway which arise after intermolecular oxidation-reduction and cleavage of the phosphoester (McGilvery, 1983). Fig.40 illustrated this scheme.

A close look at this figure suggested that 'turning on' the glucan biosynthetic pathway also stimulated the intermolecular rearrangement that led to the formation of molecules of inositol in the cytosol. Inositol could then react with phosphatidic acid forming phosphatidyl inositol, as shown in fig. 34 above. As stated earlier, P1 was part of the phospholipid component of bimolecular membranes. Along with phosphatidylserine (PS) it constitutes 35% of the plasma membrane of *S. cerevisiae* and, even then PS was found to be a minor component of the content (Suomalainen and Nurminen, 1973). On the other hand, phospholipid content of membranes of yeast-like form of *P. pulullans* was 31% while PS + P1 added up to 10% of that figure (Ponton *et al.,* 1980).

Since the yeast morphology, as occurs in the life cycle of *S. cerevisiae,* here evolved from protoplasts of strain C12, IMI W5132A in the glucose-substrated uracil-incorporated multiionic broth, we assumed that the uridyl group was incorporated into the protoplast metabolism (Fig. 37). CTP could be formed from UTP in the presence of CTP synthase with subsequent activation of phosphoryl transferase system in the phosphate-rich multiionic broth, phosphatidic acid would interact with the high energy nucleotide giving as products activated ester group CDP-diacylglyceride and the phosphoanhydride, PPi, which in the presence of pyrophosphatase would drive the reaction to completion. On the possible reaction between the activated ester and inositol, phosphatidylinositol would result (Voet and Voet, 1995; Harris 1997). This was illustrated in fig 41i. As discussed above, two subsequent phosphorylations by kinases in the inner surface of the membrane could yield phosphatidyl 4, 5 bisphosphate ($P1P_2$) and its hydrolysis would yield two second messengers DAG and $1P_3$, a reaction possible in the presence of membrane-bound phopholipase C (Fig. 41ii).

We have further assumed that the $1P_3$ induced the release of Zn^{2+} from its store and, or its cytosolic elevation stimulated translocation of the novel PKC to membrane bound phosphatidyl serine head group, where an interaction cooperatively mediated by DAG and Zn^{2+} caused the phosphorylation and release of nPKC catalytic subunit. This in turn relocated to activate membrane-bound adenylate cyclase. The phosphatidyl turnover in systems like fibroblast, where the P1 cycle operate is quite high even though DAG has a transient life; this ensures continuity in signal transduction and a possible amplification of an elicited physiological response. In our study with strain C12, IMI W5132A, we proposed that terminal yeast budding was part of such response. This meant that a sustained event of stimulation of the mitogenic process occurred, hence, leading to cell proliferation. Observation showed that it was not all the protoplasmic units that converted to the yeast form in uracil-incorporated medium. Ditto in the thymine-enhanced medium where the protoplasts were actually more numerous than it was in the uracil-favored medium. Perhaps a slower rate of conversion of the DNA-requiring pyrimidine base to RNA– obligatory-uracil and subsequent utilization in generating ligand species for activated substrates in the metabolic route for the yeast enhancing physiology, could be attributed. Of note here was the generation of (i) activated glucose, UDP-G, the substrate for glucan synthase (ii) activated phosphatidate, CDP-P and activated inositide CDP-inositol, which are substrates for phosphatidylinositol generation, so important in creating second messengers and signal transduction.

Apart from signal transducing function here discussed, it has been shown that phospholipids form part of the structural components of cell envelopes of terminal budding yeast cells, multipolar budding yeast-like cells, and mycelia (Dominguez *et al*., 1978; Ito *et al*., 1982; Kerridge *et al.,* 1976; Hunter and Rose, 1972; Ponton *et al.,* 1980; Suomalainen and Nurminen, 1973). Phospholipids are for the most parts constituents of the plasma membranes as shown in *S. cerevisae* (Suomalainen and Nurminen, 1973), but in

all microorganisms phosphatidylcholine and phosphatidylethanolamine are quantitatively more important structural phospholipids (Zubay *et al*., 1995). Although phosphatidylserine is indispensable in the activation of protein kinase C (Kikkawa *et al*., 1985), it constitutes a minor part of the membrane of *S. cerevisiae,* which include phosphatidylinositol (Suolamainen and Nurminen, 1973). While phosphatidylserine and phosphatidylinositol were found to be part of the phospholipid components of the plasma membrane of terminal budding yeast cells of *C. albicans,* they were completely absent in the mycelia form (Kerridge *et al*., 1976).

The knowledge that phosphatidyltinositol could be restricted to membranes of yeast cells and the attributed role of exogenously supplied inositol in inducing terminal budding yeast cells in glucose-substrate multiionic broth, in contrast to the mycelia form induced in control experiments (Omoifo, 1997), lend credence to our theory on the occurrence of second messengers and their signal transducing ability in stimulating genetic and physiological processes that led to the yeast morphology.

x. Second messengers, protein kinases and early gene concept

The *de novo* second messengers implicated in the growth of strain C12, IMI W5132A included $1P_3$, DAG and cAMP. Several standard texts, including those by McGilvery (1983) and Voet and Voet (1995), and a review by Lalli and Sassone-Corsi (1994), have provided insight into possible role of cAMP in cellular metabolism. Elevation of its concentration in the cytosol leads to its cooperative binding at two sites of the regulatory subunits of PKA, which in turn, release the catalytic subunits (monomers). These migrate from their anchor sites in the cytosol into the nucleus where they phosphorylate specific serine (ser) or threonine (thr) residues of their nuclear protein substrates, the activators, which act by binding to cAMP response elements CREs. In this way, cAMP–activated PKA modulates nuclear factors, which bind to DNA sequences in the promoter regions of structural genes of enzymes induced by cAMP.

The role of Ca^{2+} ions in activating protein kinases is well known. For instance, in glycogen metabolism, Ca^{2+} ions activate the oligomeric phosphorylase kinase by combining with the calmodulin subunit at two high affinity binding sites on each of the two globular domains where the ions are octahedral coordinated; the binding effect causes an extensive conformational change, thus exposing the hydrophobic patch which is methionine (met) - rich and this, in turn, combines with the catalytic subunit. A phosphorylase kinase, activated, will then catalyze the phosphorylation of glycogen phosphorylase, the enzyme that modulates glycogen breakdown. Ca^{2+} ions also play important role in modifying and activating the glycogen biosynthetic enzyme, glycogen synthase. The central role of Ca^{2+} in enzyme modification has been emphasized (Voet and Voet, 1995). This was shown in fig. 42. Voet and Voet (1995) added Ca^{2+} to the list of second messengers in mammalian systems. Other second messengers are $1P_3$, DAG and cAMP. The role of Ca^{2+} in mitogenic stimulation has also been emphasized (Berridge, 1987).

The old literature on yeast-mould dimorphism showed the central role of zinc in the conversion process. Bartnicki-Garcia and Nickerson (1962) did not only restore the magnitude of growth of *M. rouxii* to the pre-EDTA inhibited level, but also moderated transformation of multipolar budding yeast-like morphology. On the other hand, Yamaguchi (1975) showed that there was inhibition of RNA synthesis and pyrophosphate accumulation and, remarkably, filamentous growth when *C. albicans* was cultivated in zinc-deficient medium in contrast to the terminal budding yeast morphology in zinc-replete medium.

The effect on RNA synthesis, to us, was not surprising because Zn^{2+} ion has been shown to be an essential component of transcriptional activators of several structural genes. In galactose metabolism, GAL 4 gene product, a multifunctional protein, on being free of repression by that of GAL 80 as the latter combines with an inducer or available galactose, and as possibly occurred in our multiionic broth exogenously supplied galactose (Omoifo, 1997), acted as an inducer thereby making GAL 80 gene product inactive (Zubay *et al.,* 1995) hence diffused from its chromosome and translocated to chromosome XI where it binds to a 17 base pair sequence at DNA binding site (this is within 100 base pair upstream from TATA box of the promoters). It then activates the transcription of Leloir enzymes from GAL 1, GAL7 and GAL 10 respectively galactokinase, galactose-1-phosphate uridlyl transferase and UDP-galactose –4-epimerase and additionally GAL 2 gene product, galactose permease, from Chromosome XII (Abeles *et al.,* 1992, Zubay *et al.,* 1995). The outstanding fact here is that GAL 4 gene product, an 881-protein residue contains Zn^{2+} finger motifs. Residues 1-65 of its construct harbor 3 distinct modules that further heighten its affinity with the DNA main groove during interaction. Voet and Voet (1995) explained that a compact Zn^{2+} liganding domain, which binds specific sequence of DNA 8-40, has two Zn^{2+} ions tetrahedral coordinated by 4 histidine and 4 cysteine residues, bond formation being respectively at the imidazole

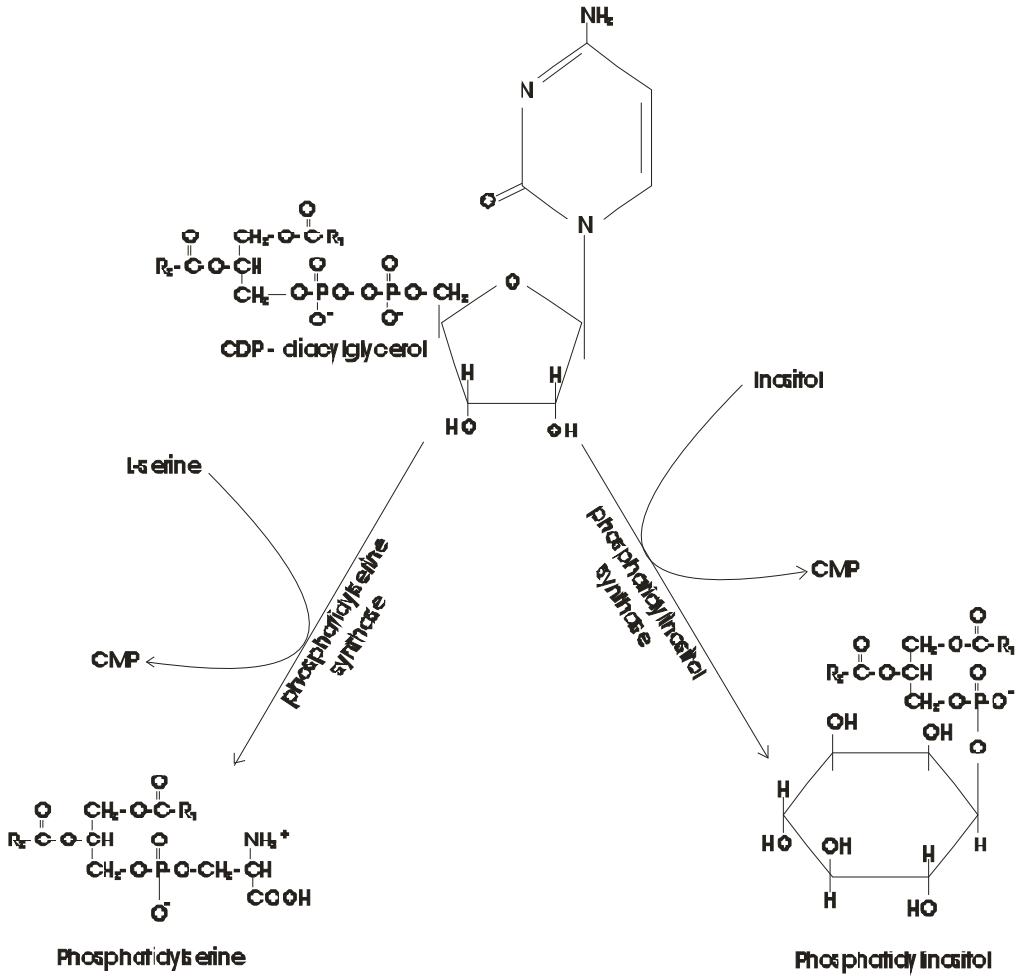

Fig. 41i: Reaction that converts CDP - diacylglycerol to phosphatidylinositol in eukaryotic cells.

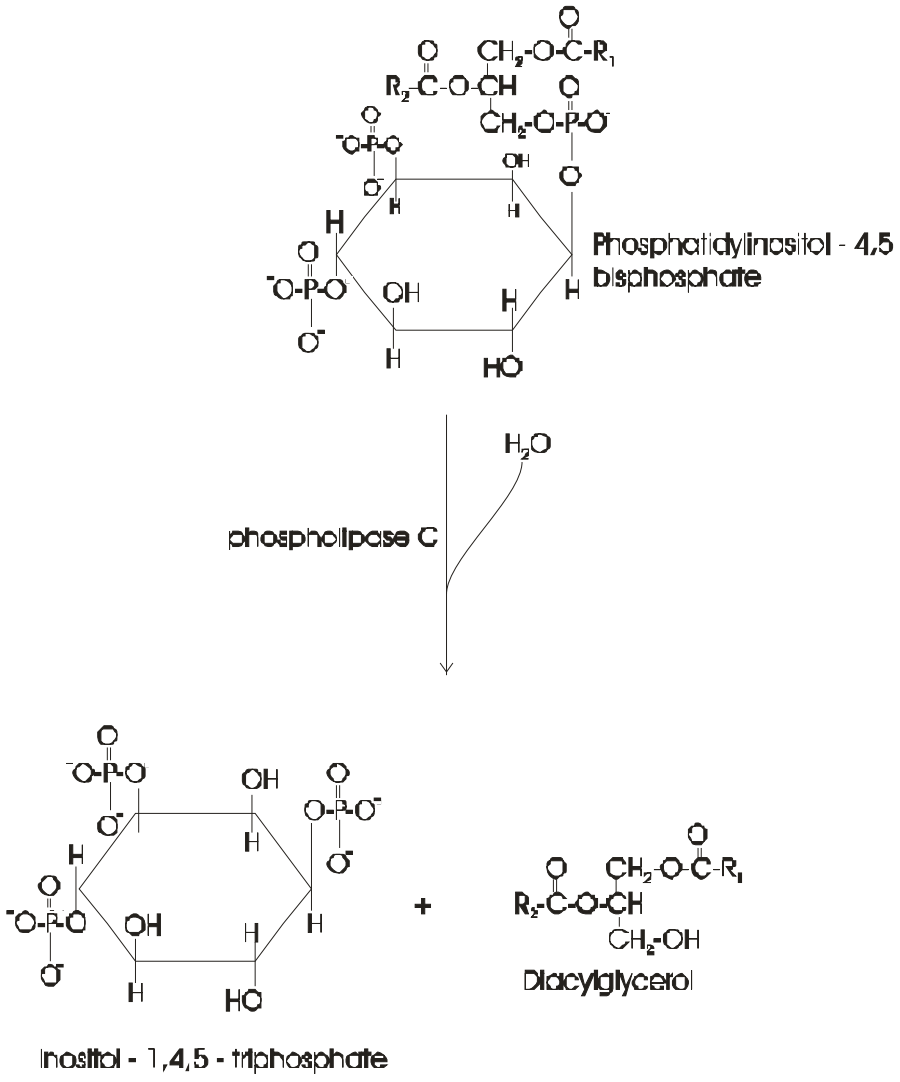

Fig. 41(ii): **Phospholipase C degradation of phosphatidylinositol - 4,5 - bisphosphate.**

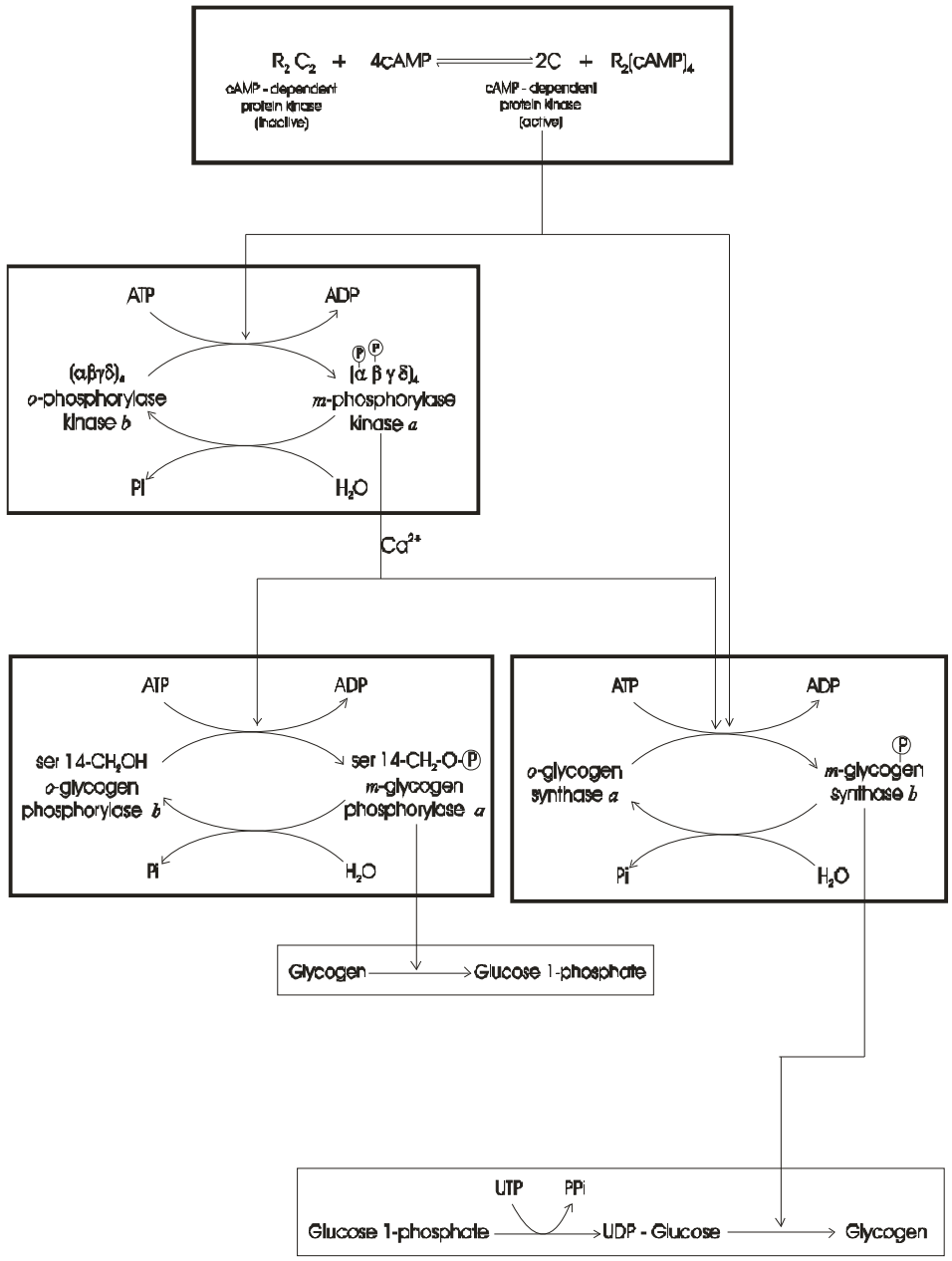

Fig. 42: Scheme showing the central role of Ca^{2+} in enzyme modification for glycogen metabolism. Modified from Voet and Voet (1995).

and thiol branch chains, but two of such cyteine residues also ligate the two Zn^{2+} ions to form a binucleate cluster. The linker module residue 41- 49 connects this to a short helical dimmer 52-64. This was represented in fig. 43. The N- terminal helix at the first module of this polypeptide inserts into the glutamine-rich region flanking the phosphorylation box of the cAMP response element binding CREB protein and there activates the transcription process. Thus, at elevation of cytosolic cAMP, PKA catalytic unit triggers the phosphorylation of a serine residue at position 113 of CREB protein. This will cause a conformational interaction with the genes' transcription process (Lalli and Sassone- Corsi, 1994).

Since sporangiospores of strain C12, IMI W5132A were simultaneously converted to mycelia structures and terminal budding yeast cells in galactose-substrated zinc- incorporated multiionic broth (Omoifo, 1997), it was suggested that the generation of second messengers led to signal transduction that possibly elicited GAL 4 gene product, a polypeptide transcription factor with zinc finger domains which activated the Leloir enzyme transcription machinery. Zinc ion, as assumed in this discourse, was involved in creating (i) Zn^{2+} finger motifs in novel PKC structural stability (component of the regulator domain) and its intracellular migration and (ii) Zn^{2+} finger motifs in GAL 4 gene product and its transchromosomal interaction. Therefore, its intrinsic manifold roles in intracellular signal transduction could not be overemphasized.

With the generation of glucose 1-P following Leloir enzyme activities, a possibility existed for activation of glucose using the salvaged phosphoanhidride UTP as ligand, UDP-glucose. It is well known that the physiological role of cAMP is exerted through kinase (Voet and Voet, 1995). If we look at fig. 42, the involvement of Ca^{2+} perhaps called for the activation of glucose using ATP as co substrate. Since in the yeast morphology, early requirement was probably not for the storage product, glycogen, rather the cytoskeletal structure, glucan, the involvement of Zn^{2+} coupled with structure of enzymes of the cAMP-induced pathway, would perhaps call for different co substrate. Thus GTP has been found to be the high-energy nucleotide and co substrate for the catalytic activity of glucan synthase, the β 1, 2, glucan biosynthetic enzyme (Cabib *et al.*1988). This probably accounted for the occurrence of terminal budding yeast cells in galactose- substrated multiionic broth.

In fig. 44, we proposed a scheme indicating the central role of Zn^{2+} in enzyme modification in the utilization of maltose substrate for yeast morphology induction. The biomass of strain C12, IMI W5132A increased 115.276 fold following incorporation of uracil into the medium of growth (Omoifo, 1997).

In the system apparently operating in strain C12, IMI W5132A, the principle of positive control appeared to be the driving force for initiation of, and transcription of, genes thus yielding proteins, which prompted reactions that gave expression to physiological processes. For instance, trans-acting GAL 4 protein presumably interacted positively with CREB protein in the promoter region of the DNA thus allowing the release of RNA polymerase II which then transcribed genetic information into individual mRNA specific for each gene-GAL 7 and GAL 10 – in the *cis* strands of chromosome XI but complimentary strand of GAL1, and also GAL 2 on chromosome XII. The gene products, which were Leloir enzymes were then used for galactose metabolism (Abeles *et al*., 1992; Zubay *et al.,* 1995). Therefore it might be right to conclude that incorporation of inositol into glucose- substrated multiionic broth triggered inositide cascade, which in turn stimulated the cAMP-protein kinase pathway that activated the gene system formatting physiological development, which endowed particular morphological expression.

As CREB mediated gene expression in response to elevated cellular cAMP (Xing *et al.,* 1996), it was suggested that CREB was the promotional element at play in the studies of Larsen and Syphered (1974) and Paveto *et al.* (1975) in which sporangiospores were transformed to multipolar budding yeast-like cells in response to exogenously applied cAMP. It was thought that the cAMP response pathway caused translocation of PKA to the CREB domain where it interacted with the CREB phosphorylation box domain, an event that induced conformational change in the protein which in turn exposed the DNA flanking glutamine rich domain for further interaction with the machinery of transcription (Lalli and Sassone-Corsi, 1994). Chriva and colleagues, cited by Lalli and Sassone- Corsi (1994) actually isolated a protein with two Zn^{2+} finger motifs and a PKA phosphorylation site that gave premium to this notion.

Xing and colleagues (1996) noted that CREB also regulated cellular response to growth factor stimulation, by activating the CREB's transcriptional potential when phosphorylation occurred at a specific amino acid residue, serine at position 113. The specific kinase responsible for the critical phosphorylation was CREB kinase. Xing and his colleagues in their study purified this kinase to homogeneity from TPA-, a growth factor - treated human erythroleukemia cell line K 562 cells. But peptide sequence comparison showed that it had absolute identity with ribosomal protein S6 kinase 2, RSK2, which was

chromatographically detected to be physiologically active even *in vivo*. Further experiments in the study showed that RSK2 endogenously executed phosphorylation of recombinant CREB-GAL 4 *cos* cells precisely at serine –113 in a growth factor stimulated process.

In their extensive study Xing and colleagues showed that the pathway for signal transduction from extracellular growth factor cascaded through MEK (the kinase that phosphorylates and activates MAPK at serine 218/222), succeeded by MAPK mitogen-activated protein kinase and subsequently, RSK2. According to Voet and Voet (1995), MAPK structurally resembles PKC. Perhaps PKC would perform similar functions as MAPK. This suggestion was supported by the fact that PKC could also effect phosphorylation at CREB protein of serine residue at position 113 (Lalli and Sassone-Corsi 1995).

Since we have implicated the novel PKC with Zn^{2+} finger motif in signal transduction during the cultivation of strain C12, IMI W5132A in buffered multiionic broth without extracallular growth factor incorporation (above), we suggested that following activation by exogenously supplied Zn^{2+} and intramembrane-derived DAG, the novel PKC could stimulate and activate RSK2. Ribosomal protein S6, an essential component of the 40S ribosomal subunit, and preferentially located for RNA binding role, on being phosphorylated would drive forward the interaction between mRNA and 40S ribosomal subunit (Berridge, 1987). Thus, the preceding kinase cascade could stimulate the translation of, and activation of protein synthesis of, the early gene products. Such gene products could be greater in quantity and variety in yeast cells in comparison with hyphae, which, as Larsen and Sypherd (1979) showed in *M. racemosus*, had lesser degree of phosphorylation at the S6 subunit. In our system, this perceivably occurred at P_g-G_2 subphases of the conjugal *cdc* (Fig. 107), where subspecies α and δ polymerases were thought to be the committed species to DNA synthesis, as occurs in *S. cerevisiae* and other eukaryotes (Fry and Loeb, 1986).

The P_g subphase, it would appear, was critical in the differentiation of organelles and cytoskeletal structures that elaborated the direction of a particular physiology. Threshold levels of proteins from the *fos, myc, actin*- and *tubulin* - transcribed gene, promoted by Zn^{2+} finger CREB protein, would thus accumulate to specify the physiology that prepared the protoplast at the G_2 subphase for the proceeding mitotic event, a process that could then give rise to cell proliferation. A subsequent study by Larsen and Sypherd (1980) showed that the phosphorylation of S6 subunit was elevated and this correlated with the growth rate of the organism, *M. rouxii*, which occurred in the yeast-like form. Thus, if the levels of specific gene products, like glucan synthase and chitin synthetase, respectively, were elevated, they could therefore construct and impose a yeast and mycelia form on the assumed physiology (see Fig. 26).

The critical role played in enzyme modification by Ca^{2+} is well known. It acts through the intermediacy of calmodulin and it is an intracellular signal transducer (Charp and Whiteson, 1982; McGilvery, 1983; Zubay *et al.,* 1995; Voet and Voet, 1995). Ca^{2+} ion is also credited with a regulatory role in *fos* transcription and S6 phosphorylation (Berridge, 1987). Zn^{2+} ion, which like Ca^{2+} ion, is electrogenically transported through cellular membranes via carrier proteins, as well as shares responsibility for Na^{+}-H^{+} regulation (Zhuang *et al.,* 1995), might similarly function in enzyme modification (Fig.44).

This treatise has generally implicated Zn^{2+} ion in (i) metallo-protein motifs (ii) intracellular ionic transport and (iii) agonists. Hence, we also adduce its involvement in the regulation of *fos* transcription and S6 phosphorylation during the cultivation of strain C12, IMI W5132A in maltose-substrated buffered multiionic broth. Figs. 45 and 46 are interpretative schemes for possible integrated yeast cell formation from protoplasts of, after cell wall lyses of sporangiospores of, strain C12, IMI W5132A respectively, in galactose – substrated and maltose- substrated multiionic broths.

D. EXPERIMENTAL: EFFECT OF GROWTH INHIBITORS ON INDUCED YEAST MORPHOLOGY OF STRAIN C12, IMI W5132A

The precepts elaborated in the foregoing theory challenge some of the concepts developed over the last century. Most important of all was that inhibition of respiration was the key event that induced morphological change in dimorphic fungi. If we consider respiration to proceed in the following steps (i) glycolysis (ii) TCA, that is, degradation of pyruvic acid to CO_2 through a series of organic acids, to ATP, then blocking any of the steps or, a reaction step thereof, would possibly prevent completion of this physiological process. Such experimental blockage would provide evidence to validate, or otherwise, the theory on respiratory reposting of morphological change. As a higher proportion of respiratory ATP was derived at step 3, inhibiting this terminal part by azide, a known respiratory inhibitor, which could abrogate redox reactions of electron transport proteins, cytochromes, by the ligand azide-ferriprotoprophyrin IX bond, could be a test case.

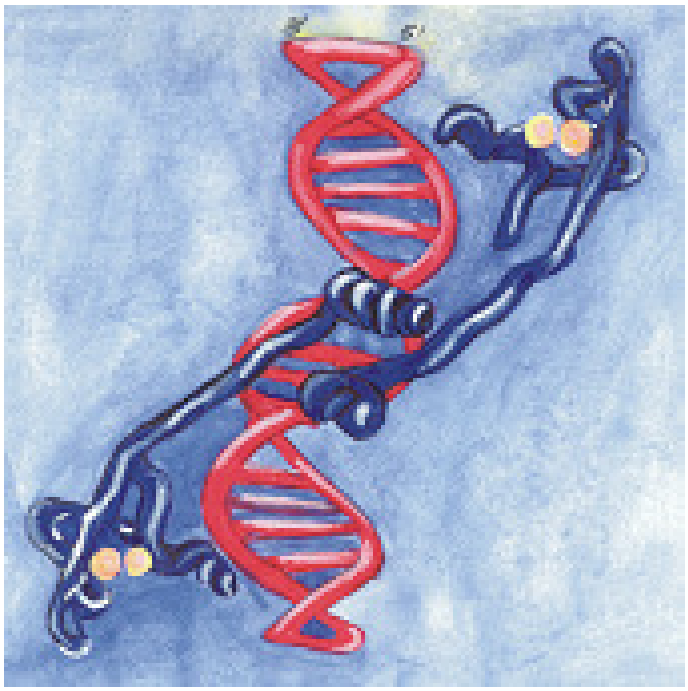

Fig. 43:Artistic impression of the structure of GAL 4 DNA binding domain in complex with a palindromic 19-bpDNA, presented in Voet and Voet 1995, pp. 1163. It was redrawn so as to emphasize the positions of Zn^{++} - finger motifs (yellow balls) in DNA – protein interaction.

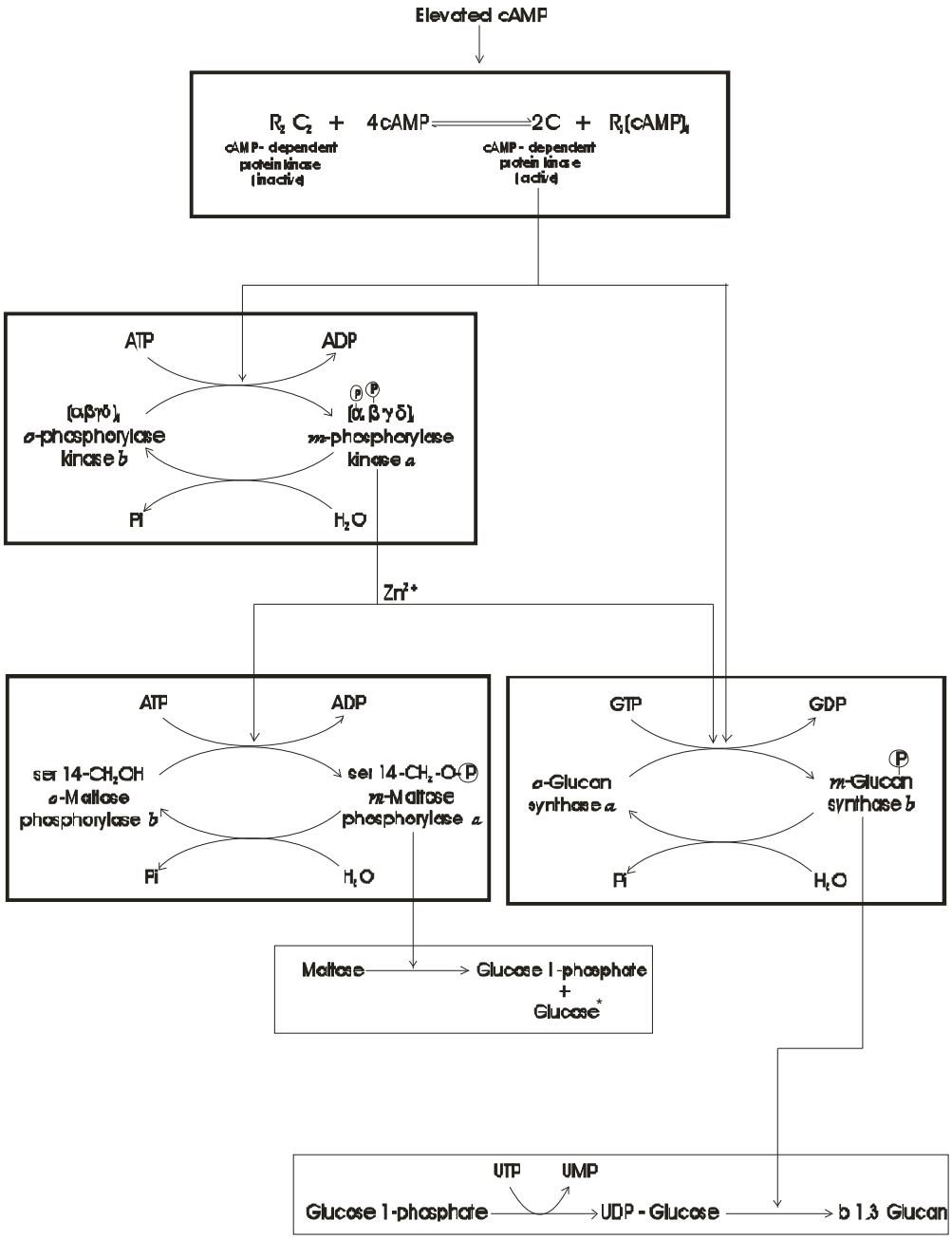

Fig. 44. An interpretative scheme showing the central role of Zn^{2+} in enzyme modification for maltose metabolism in the presence of exogenously supplied uracil. This scheme was modelled on Fig. 42. * It was assumed that the glucose moiety of the disaccharide was then primed for the glycolytic process.

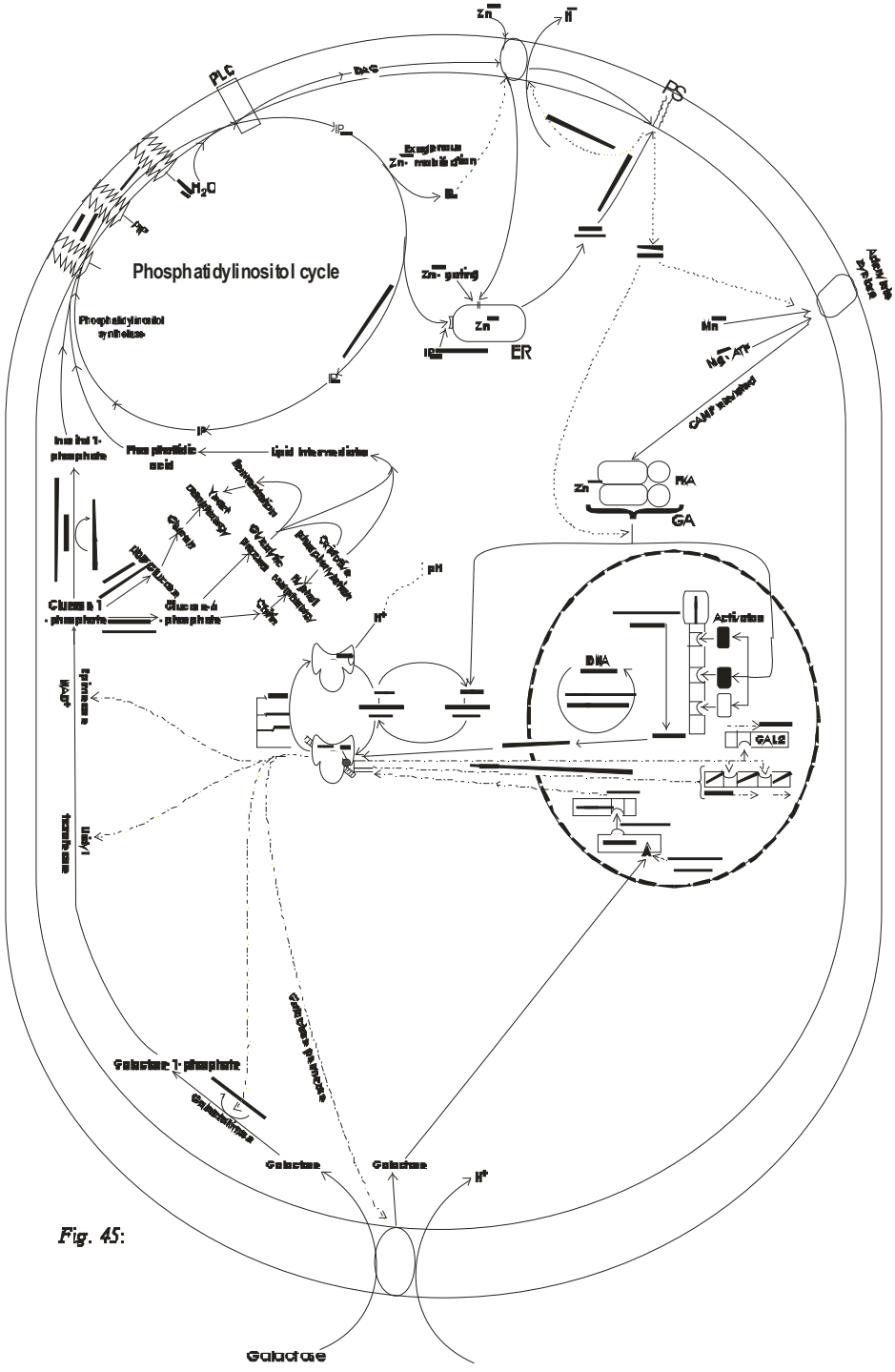
Phosphatidylinositol cycle
Phosphatidylinositol synthetase
Zn- gating
ER
PLC
DAG
PKC
Zn- mobilization
Mn
Mg-ATP
cAMP activated
PKA
GA
Activation
DNA
GAL2
pH
Galactose 1- phosphate
Galactose
Galactose
Galactose

Fig. 45:

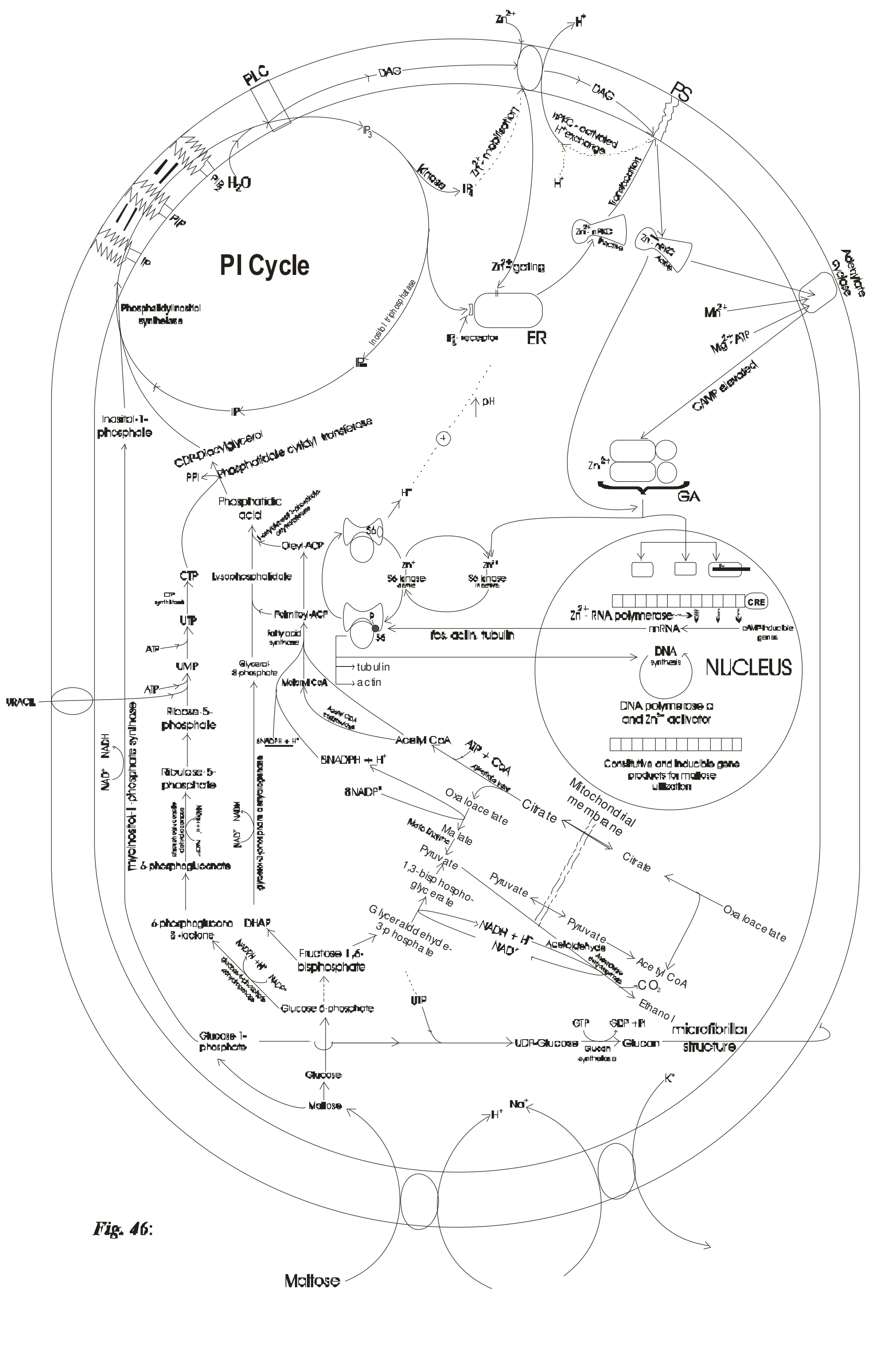
PLC
DAG
PS
PI Cycle
Kinase
Phosphatidylinositol synthetase
Inositol triphosphatase
ER
Adenylate cyclase
cAMP elevated
GA
NUCLEUS
RNA polymerase
mRNA
CRE
fos, actin, tubulin
tubulin
actin
DNA synthesis
DNA polymerase α and Zn activator
Constitutive and inducible gene products for maltose utilization
Mitochondrial membrane
Inositol-1-phosphate
CDP-Diacylglycerol
Phosphatidate cytidyl transferase
Phosphatidic acid
Lysophosphatidate
Oleyl-ACP
Palmitoyl-ACP
Malonyl CoA
Acetyl CoA
Glycerol-3-phosphate
CTP
UTP
UMP
URACIL
Ribose-5-phosphate
Ribulose-5-phosphate
6 phosphogluconate
6 phosphoglucono δ-lactone
Glucose 6-phosphate
Fructose 1,6-bisphosphate
DHAP
Glyceraldehyde-3-phosphate
1,3-bisphosphoglycerate
Pyruvate
Malate
Oxaloacetate
Citrate
Acetaldehyde
Ethanol
Glucose 1-phosphate
Glucose
Maltose
UDP-Glucose
Glucan
microfibrillar structure
Glucan synthetase
Maltose

Fig. 46:

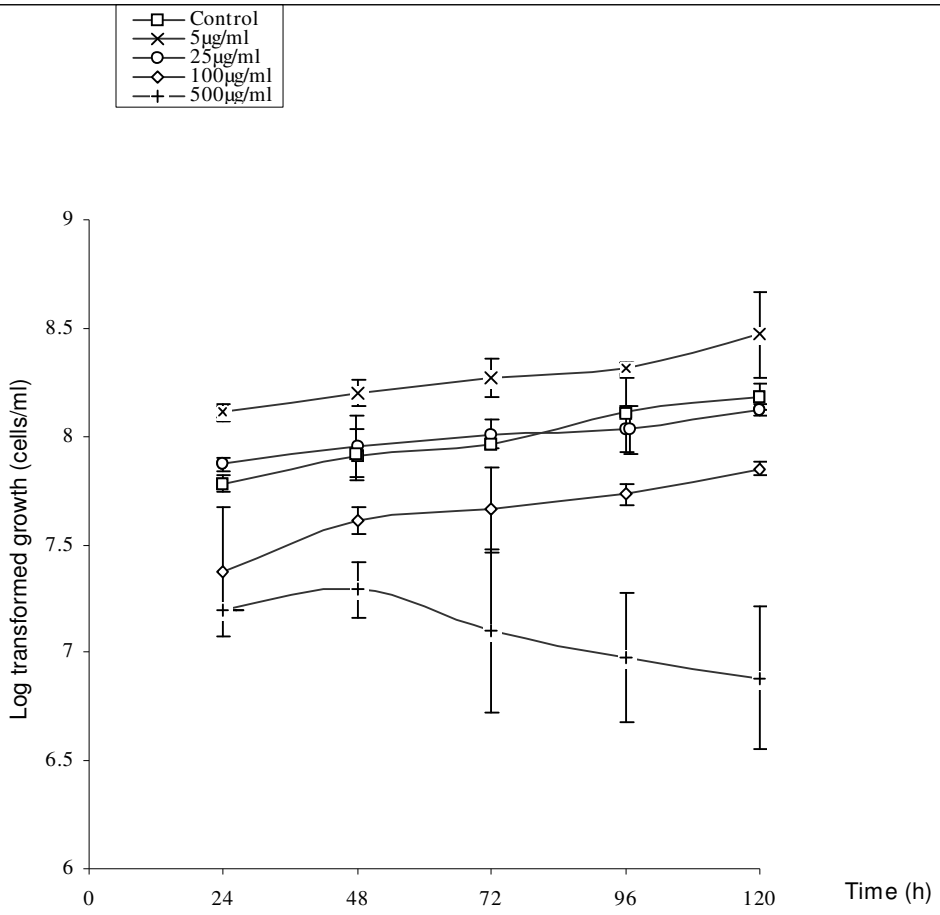

Fig. 47 :.Effect of 2,4-D on the growth of strain C12 W5132A in maltose-substrated uracil-incorporated multiionic broth pH 3.5. Each point represents means of triplicate determinations plus x10 std. dev.

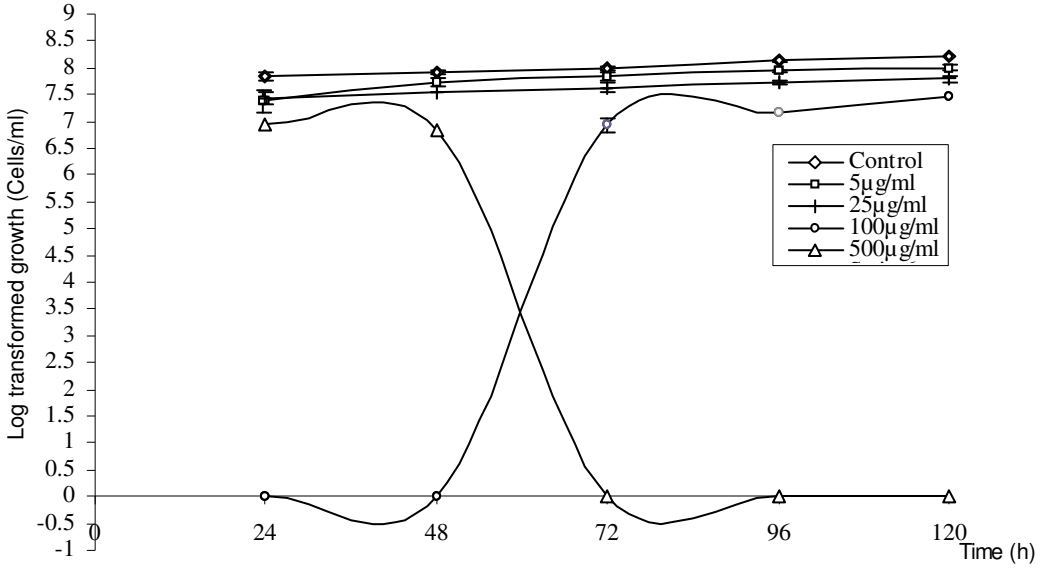

Fig. 48: The effect of glyphosate on the growth of strain C12 W5132A in maltose-substrated uracil-incorporated multiionic buffered broth, pH 3.5. Each point represents means triplicate determinations plus x 10 standard deviation.

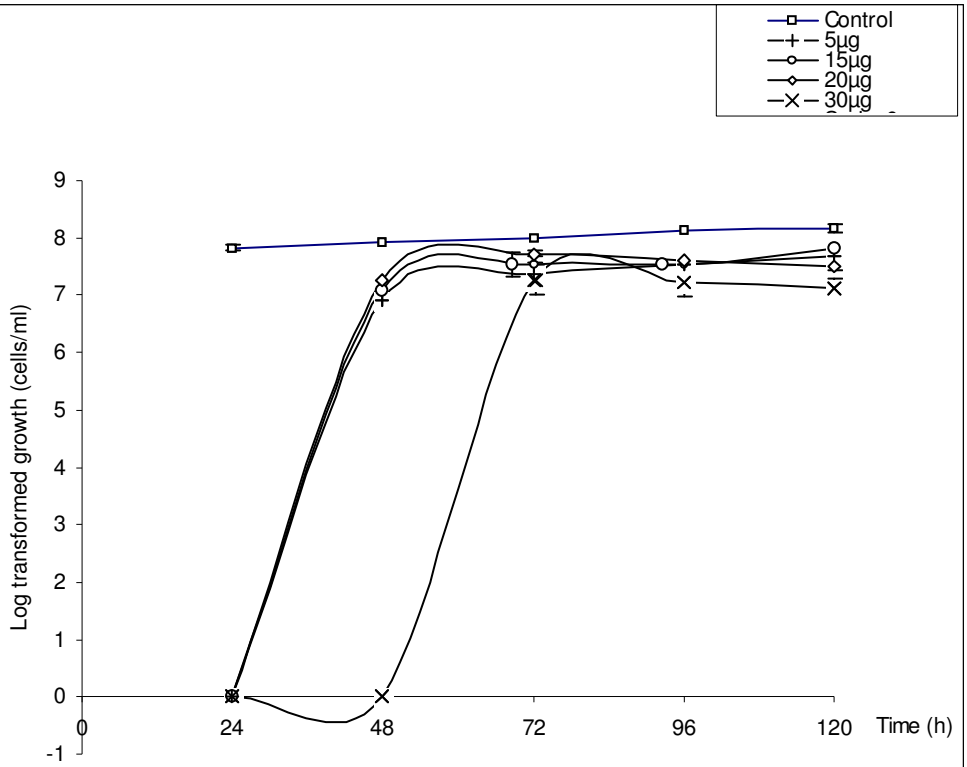

Fig .49 : Effect of sodium azide on growth of strain C12 W5132A in maltose-substrated uracil-incorporated multiionic buffered broth pH 3.5. Each point represents means of triplicate determinations plus x10 std. dev.

Glyphosate blocked the incorporation of labeled uracil into nucleotides (Breche, 1976 cited in Ashton and Crafts, 1981). This was particularly interesting, since it was presumed that strain C12, IMI W5132A synthesized nucleotides through salvaged routes in the presence of exogenously supplied uracil. Therefore, limiting the ability of strain C12, IMI W5132A to incorporate the pyrimidine base into nucleotides by using glyphosate would probably play a decisive role in cellular metabolism and, or morphogenesis.

Treatment of cells with 2,4 dichlorophenoxyacetic acid (2, 4-D)- activated RNA polymerase had direct effect on mRNA activity and subsequently stimulated the biosynthesis of RNA and proteins which gave rise to cell proliferation (Ashton and Craft, 1981; Shanon and Hansen, 1962). Shanon and Hansen (1962) found that Corn mesophyl tissue increased 3-fold following 2, 4-D treatment. This possibly arose from an increase in ribonuclease activity, which directly or indirectly activated limiting enzymes (Key, 1964). While low concentrations of the auxin have been found to be stimulatory, higher levels were inhibitory (Ashton and Craft, 1981). Lag time study by Key and Shanon (1964) showed that at 5µg/ml 2, 4-D there was 71% increase in uridine incorporation into RNA of elongating cells in contrast to control experiments but this decreased to 67% and 57%, respectively, as the level of 2,4-D increased to 25µg/ml and 100µg/ml. On the other hand, Okay and Gaines (1996) found an extended duration of lag phase and growth inhibition of phytoplanktons with an application of 100µg/l 2,4-D.

Thus, if terminal budding yeast morphology was the recognized pattern of growth of strain C12, IMI W5132A in specified minimal medium, various degrees of treatment with 2, 4-D, glyphosate and azide could relate inherent changes in the pattern physiology manifesting time- course profile and morphology.

Plan of experiment

A population of sporangiospores, from seven- day old agar cultures of strain C12, IMI W5132A, was used. It was obtained by pouring sterile distilled water over slant cultures in boiling tubes and dislodging spores with sterile glass rod. The suspension in each tube was decanted into a 250ml sterile conical flask. The volume was decanted into centrifuge tubes and washed at 3,000g temp 25^{o}C for 7min. It was subsequently washed with two changes of sterile distilled water. Spore density, 10^{6}/ml, was obtained as previously described (Omoifo, 1996b). An Iml of this suspension was inoculated into a 1% maltose-substrated, uracil- incorporated multiionic broth buffered at pH 3.5 (Omoifo, 1997). This was incubated at 20^{o}C ambient, for 5 days. Growth count and morphological examination was done at 24h intervals. From this preliminary experiment, data were obtained indicating the morphology and growth pattern of the microorganism. Subsequently the main study was conducted with the concentrations of 2,4-D: 0, 5, 25, 100 and 500µg/ml; glyphosate: 0, 5, 25, 100 and 500µg/ml; sodium azide: 0, 5, 15, 20, and 30µg/ml. Also examined was the possible effect of inhibitory concentrations of glyphosate and azide in counteracting 2, 4-D stimulation of growth, on concurrent application.

Results

***Effect of 2, 4-D on growth and morphology of strain* C12, IMI W532A**

The microorganism strain C12, IMI W5132A grew in broth with or without the incorporation of 2,4-D and without a lag phase. The induced cells had similar morphology in both treated and control broths. This was terminal budding yeast form. However, differences occurred in the growth pattern. Optimal growth in 5µgml 2,4-D- incorporated medium was distinct (Fig. 47); this indicated a stimulatory effect of the chemical at this concentration. Growth at 25µg/ml 2,4-D was apparently similar to that of control. With further increase in concentration, growth was suppressed. While control and 2,4-D incorporated tests showed relative accumulation of cell number with time, it declined after 48h at the highest level of growth factor incorporation.

***Effects of Glyphosate on growth and morphology of strain C12* IMI W5 132A**.

Optimal growth in control cultures was only better than that incorporated with low concentration of glyphosate although morphological expression of induced cells was similar, that is, terminal budding yeast cells. This, however, indicate that the strain was able to adapt to the environment. The growth pattern was shown in fig. 48. A lag phase of 72h duration accompanied the growth of the microorganism with

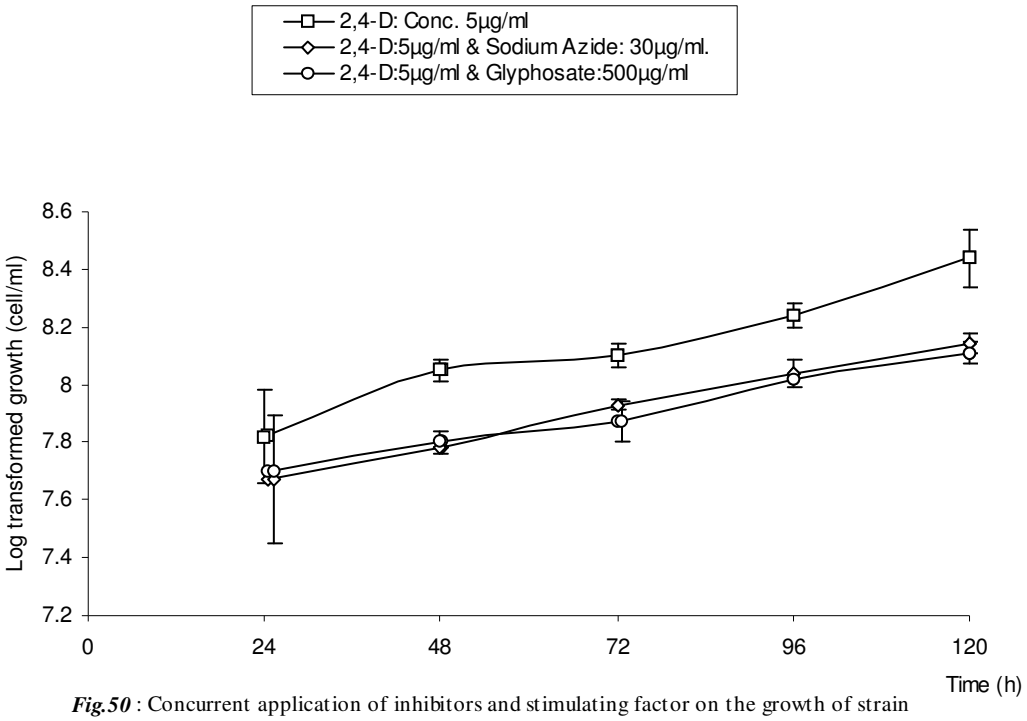

Fig.50 : Concurrent application of inhibitors and stimulating factor on the growth of strain C12 W5132A in maltose substrated uracil-incorporated multiionic buffered broth pH 3.5. Each point represents means of triplicate determinations plus x10 std. dev.

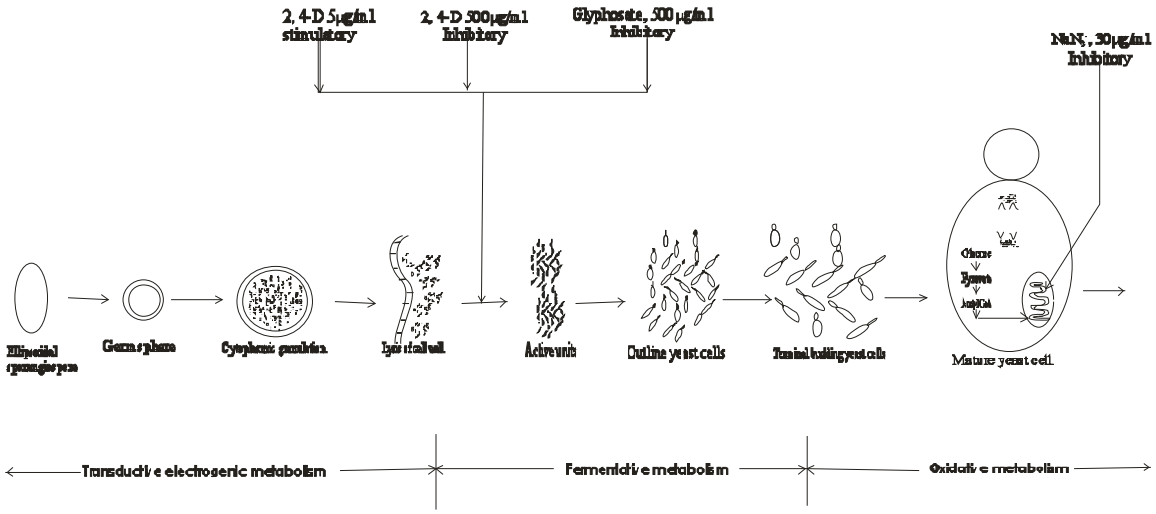

Fig. 51: Scheme showing possible steps where stimulatory and inhibitory concentrations of 2, 4-D, glyphosate and azide were active during sporangiospore-yeast transformation of strain C12, IMI W5132A cultivated in buffered multiionic broth at 20°C, pH 3.5.

100µg/ml incorporation. In other words, a length of time was required for cell biochemistry to overcome the increased concentration. Another distinction was that two types of morphologies were induced and these included conidia, which were scanty, and terminal budding yeast cells, which predominated. At the highest concentration tested, growth spheres occurred within the first 48h and thereafter only protoplasts were encountered. But these were never counted.

***Effect of NaN_3 on growth and morphology of strain* C 12, IMI W5132A**

In contrast to control test, growth in azide incorporated media showed a lag of 48h, except the highest level tested which had a longer lag phase. This was shown in fig. 49. Thus, while the microorganism required time to adapt to azide incorporated environment, such became prolonged at 30µg/ml. Azide diminished the maximum population of strain C12, IMI W513A. However, morphological expression in this set of experiments was not different form that exhibited in control cultures, which is, terminal budding yeast cells.

***Effect of concurrent application of stimulatory concentration of 2, 4-D and inhibitory concentrations of glyphosate and azide on growth and morphology of strain* C12, W5132A**

Time-course plots of microbial numbers in cultures with concurrent application of stimulatory concentration of 2, 4-D and inhibitory level of the azide or glyphosate did not show pattern difference. But optimal growth of induced cells in stimulated cultures was distinct (Fig. 50). This indicated that the microorganism in the presence of the stimulatory level of 2,4-D was able to overcome the repressive effects of the inhibitors at the concentrations used. However, the morphology exhibited was terminal budding yeast cells.

Discussion

The present results demonstrated that low concentrations of 2,4-D stimulated the growth of *D. diastaticus* strain C12, IMI W5132A. This is in agreement with the results obtained by Key and Shanon (1964) who worked on soya bean, *Glycine max,* seedlings and Kay and Gaines (1996), on the diatom, *Phaeodactylum tricornutum.* It has been shown that the main effect of 2,4-D is exhibited by increasing RNA polymerase activity with consequent effect on protein synthesis; this would lead to cell proliferation (Cherry, 1976; Key and Hansen, 1961; Key and Shanon, 1964; Shanon and Hansen, 1963; Key, 1964; Ashton and Crafts, 1981). Mann and Pu (1968) in another vein showed that it acted by elevating lipid biosynthesis. Perhaps this process was also elected during the growth of strain C12, IMI W5132A.

In the study of Pieper *et al.* (1988), it was shown that the bacterium, *Alcaligenes eutrophus* JMP 134, which was grown on 2,4-D, 4-chloro-2-methyl phenoxy acetic acid (MCPA) and 2-methyl phenoxy acetic acid (MPA) constitutively produced the enzyme, 2,4-D monooxygenase and this was utilized in its metabolism that enabled it to obtain carbon-energy from the substrate. In the case of 2,4-D, the enzyme cleaved the ether bond thus leaving 2,4-dichlorophenol and acetate as the byproduct. If we assumed that 2,4-D monooxygenase activity was induced in strain C12, IMI W5132A in the presence of 2,4-D, then the acetate moiety made available could be converted through condensation reactions to lipids. We showed in fig, 46 a scheme for the possible utilization of activated acetate for the production of phosphatidic acid in the pathway to second messenger generation and protein kinase cascade during the growth of strain C12, IMI W5132A. It was therefore possible that cultivation of strain C12, IMI W5132A in maltose-substrated buffered multiionic broth incorporated with 5µg/ml 2,4-D stimulated the induction of 2,4-D monooxygenase activity, which provided additional units of acetate; this would therefore enhance the activated acetate pool, and subsequently stimulated cell proliferation. However, utilization of the other catabolic product, 2,4-dichlorophenol would depend on induction of ortho-cleavage enzymes, including chlorophenol hydroxylase, catechol 1,2-dioxygenase and muconate cycloisomerase required in the ortho-cleavage pathway for 2,4-D degradation (Pieper *et al*., 1988). The study of Marriott *et al.* (2000) showed that *A. denitrificans* effectively degraded 2,4-D and other phenoxy chlorophenoxyalkanoic acids in single, binary, tertiary and quaternary combinations and this was attributed to possession of a dominant gene for encoding α - ketoglutarate-dependent dioxygenase.

The fact that no lag period was exhibited meant that the higher concentrations of 2,4-D did not prevent the commencement of any of the physiological processes during the development of yeast form from sporangiospores of strain C12, IMI W5132A. Hence, the suppression of optimum growth was interpreted as the inability of the low level inductive enzymes to cope with the high level 2,4-dichlorophenol generated. In which case, accumulation of the phenol moiety could become toxic to, and hence repress growth of, the organism. This possibly accounted for the declining trend observed at the 500µg/ml concentration.

Evolution of the yeast morphology sequentially involved isodiametric growth of sporangiospore into growth sphere, lysis of cell envelope and release of cytoplasmic units, the multi-dimensional growth of these units into protoplasts and emergence of yeast form from the protoplasts. In this study, the toxic effect of 100µg/ml glyphosate caused the lag period of 72h. Brecke (1976, cited by Ashton and Crafts, 1981) found that glyphosate suppressed the incorporation of uracil into RNA in isolated bean cells. Since strain C12, IMI W5132A is a uracil auxotroph, perhaps this action was executed at the level of genomic transcription during the transformation process. That is, when uracil and other substrates were already transported into the intracellular medium of protoplasts, in a similar manner with proton-substrate symport assumed for strain C13, IMI W5132B (Omoifo, 1996b).

The primary site of inhibitory action of glyphosate is the shikimate-chorismate route (Humburg *et al*., 1989). It involves a multienzyme complex with covalently linked 3-enolpyruvyl shikimate-5-phosphate synthase, which is thus repressed from catalyzing the conversion of shikimate to 3-enolpyruvyl shikimate 5-phosphate (Humburg et al., 1989), the precursor to chorismate, the chemical that forms the central branch point for the synthesis of aromatic amino acids and secondary metabolites (Smith *et al*., 1978). Induced yeast cell walls of dimorphic fungi like *M. rouxii* (Dow and Rubery, 1977) and *B. dermatitidis* (Roy and Landau, 1972) have been shown to contain two aromatic amino acids, tyrosine and phenylalanine, just like the cell walls of *S, cerevisiae* (Power and Challimore, 1969).

Two different types of morphology occurred at the 100µg/ml glyphosate. These were conidia and yeast cells, although the latter predominated. But it meant that a few spores were converted to holoblastic conidia without lysis of the cell envelope or formation of protoplasts while the majority formed growth spheres and subsequently protoplasts from which the yeast form emerged. The high optimal growth after the lag period at this concentration meant that the culture had time to become acclimatized to the chemical and, could even have approximated the control and other growth values, given more time. The plot was different at 500µg/ml glyphosate. There was no lag phase, but growth spheres only, were counted within the first 48h of growth. Granular units and protoplasts occurred thereafter. These were the only morphological entities observed till termination of the experiments. But they were not counted. We conclude that the inhibitory effect of the chemical at this concentration was profound. Hence yeast cells could not evolve. The picture that emerged was that the inhibitory effect of glyphosate was exhibited at the second phase of growth of strain C12, IMI W5132A, when cellular metabolism was bound by the protoplast membrane and that there was proton–substrate symport, as proposed by Omoifo (1996b), which then determined the physiological direction that led to the yeast morphology.

One previous study showed that azide inhibited the growth of *D. pleomorphis* strain C13, IMI W5132B. Although only one level of the inbhibitor was used in that study, the negative generation time derived was interpreted as resulting from extenuations of the terminal budding yeast cells, which were induced from sporangiospores. Similar morphology was induced from sporangiospores of strain C12, IMI W5132A in the present study. However, time-course study showed a lag of 48h at low and intermediate concentrations of the inhibitor. The lag period extended to 72h at 30µg/ml.

Although growth was suppressed in azide-incorporated media and the plots uneven in contrast to control tests, possibly indicating physiological variation, optimal growth showed an adaptation to the growth environment. What needed to be known was whether the ability to derive energy through oxidative phosphorylation was re-enlisted in such medium? This became pertinent because from the data from azide – incorporated medium, it was deduced that the terminal budding yeast morphology was invested before commencement of oxidative phosphorylation.

Respiratory chain phosphorylation entails the oxidation of glycolysis - and the TCA cycle- generated NADH by molecular oxygen through a series of reductions and oxidations by multienzyme complexes; it also involves the sequestering of the energy released during electron transfer by ATP synthase therein utilized in driving ATP synthesis from ADP and inorganic phosphate. In the inner mitochondrion, charge cluster 111, or cytochrome bc_1 complex, containing ferriprotoporphyrin 1X prosthetic protein, catalyzes the transfer of electrons from reduced ubiquinone to cytochrome c, a heme c, also ferriporphyrin containing. This is summarized as follows

$$UQH_2 + 2Cyt\ c\ (Fe^{3+}) \rightarrow UQ + 2Cyt\ c\ (Fe^{2+}) + 2H$$

In the series, charge cluster 1V, Cu-requiring heme a containing cytochrome oxidase (Cyt a + a_3), catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen which thus forms water

$$4Cytc\ (Fe^{2+}) + O_2\ 4H \rightarrow 4Cy\ c\ (Fe^{3+}) + 2H_2O$$

It is noted that azide inhibits respiratory oxidation at charge cluster 1V thus preventing the transfer of electrons to molecular oxygen (White *et al*., 1959; Jain, 1972). In their highly elucidating report, Friend e*t al* (1980) used the discrete charge electrostatic permutations to show that azide formed a coordination complex with iron-heme, which thus cancelled out the charge on the iron atom. Therefore, Fe-N_3 binding was seen as an electrostatic interaction. The coordinate bond formation was represented thus

$$Fe+(H_2O + N_3^-) \rightarrow Fe\text{-}N_3 + H_2O$$

the ligand neutralizing the positive charge on the iron while displacing the bound water molecule. This enabled Friend and colleagues to treat the ferrimyoglobin azide electrostatic interaction with precise data and thus obtained realistic results from experiments conducted at pH 4-6 and ionic strength 0.001 – 0.010M. Since our study was conducted in multiionic broth buffered at pH 3.5, and therefore high ionic strength, we proposed that the azide inhibitory activity was not only on cytochrome oxidase, but also extended to cytochromes bc_1 and c, which like ferrimyoglobin, are heme containing. That means each group of cytochrome contained porphyrin ring, although differently substituted and coordinated with the redox – active Fe^{3+}

Recall that iron-azide binding prevents redox reactions at the level of cytochrome oxidase. Since ATP synthesis is intimately associated with charge clusters III and IV, then phosphorylation at these levels would equally be uncoupled. Despite this apparent abrogation of energy supply from oxidative phosphorylation, none of the sequences in the transformative process to the yeast morphology was prevented during the cultivation of strain C12, IMI W5132A in azide-incorporated medium. Although Fisher (1977) attributed morphological change in *M. pusillus* to azide inhibition of respiration, the particular morphology exhibited was multipolar budding yeast-like cells. This contrasted with the sequentially derived terminal budding yeast cells of strain C12, IMI W5132A obtained in this study. On the other hand, Omoifo (1996b) attributed the induction of multipolar budding yeast-like cells of strain C13, IMI W5132B to the presence of complex nitrogen source in the medium of growth. When inorganic nitrogen source was used instead, terminal budding yeast cell was the sole morphology. It was observed that the composition of Fisher's medium included a complex nitrogen source, peptone. Perhaps this was partly responsible for induction of the yeast-like morphology.

The claim that oxidative phosphorylation was inhibited during the growth of strain C12, IMI W5132A was supported by the study of Reber *et al.* (1977) which showed that ATP was not generated through respiration by *Klebsiella aerogenes* cultivated in azide incorporated medium. Yet transport activities through the mitochondria membrane were permitted. It was further shown that in strictly anaerobic conditions, energy for the transport activities was provided by ATP hydrolysis and this sustained the transport activities for up to 60% of aerobically grown cells. High phosphate bonds in such anaerobic systems were generated from proton motive force. This phenomenon could be explained by the Mitchellian chemiosmotic hypothesis. An electrochemical gradient was created across the membrane, from the region of low H^+ inside the matrix, and hence inner membrane negative potential, to the region of high H^+ outside, i. e. intermembrane space, and as such high positive electrical potential. The free energy of electron transport conserved by the generation of electrochemical gradient constituted the driving force for ATP synthesis.

Omoifo (1996b) showed that the pH profile during the cultivation of strain C13, IMI W5132B in glucose-substrated multiionic broth was biphasic. Simultaneously, two different morphologies of the organism, and these constituted the countable units, growth sphere and yeast cell, respectively in phases II and I, were observed. The progressive decrease in pH of the growth medium at phase I, meant that there was H^+ efflux from intracellular medium of the growth sphere until the point of inflexion when there was consequent rise at phase II. The latter represented a progressive H^+ uptake by the yeast cell. Thus, apparently, active efflux and influx of H^+ against a buffered environment created pH differentials across the

plasma membrane of each cell type. Hence, could result in the formation of electrochemical potential gradient. In such case, the energy released in electron transfer could be harnessed for ATP synthesis (Mitchell, 1967). Although morphological and biochemical differences occurred between strain C13, IMI W5132B and strain C12, IMI W5132A (Omoifo 1996a), the same fundamental principles were thought to be involved in sporangiospore – yeast transformation of the two strains, except the requirement for auxotrophy and a disaccharide substrate by strain C12, IMI W5132A (Omoifo, 1997). Thus, the operation of Mitchellian proton pump mechanism would ensure a proton-substrate symport and the availability of maltose intracellular. This therefore stimulated physiological processes incorporating electrogenic energy transduction, second messenger generation and protein kinase cascade, and glycolysis and cytoskeletal biogenesis. These were represented by the schemes shown in fig. 35, 37, 40, 41, 43, 44, and 46, the final result being the terminal budding yeast morphology.

The lag exhibited in azide-incorporated medium was a reflection of delayed evolvement of yeast cells from protoplasts of strain C12, IMI W5132A. Although initial energy transduction through oxidative phosphorylation has been discounted, early mitochondria biogenesis in yeast formation was considered critical, being paramount in generating acetyl CoA, necessary for the biogenesis of lipid, which is in the pathway to phosphatidate formation. This was illustrated in fig. 39. If, as depicted in fig. 46, activated phosphatidate in the form of CDP-diacylglycerol was substrate for second messengers, then it was critical in generating functional linkages that activated the organism's DNA and, hence produced structural proteins that induced the yeast morphology. What this meant was that impairing biogenesis of, or functioning of the mitochondrion, as did the respiratory inhibitor, would inhibit development of the yeast form. The declining cell number, after an initial rise at the higher azide concentrations possibly reflected a more profound effect on mitosis and cell division after the yeast form evolvement. The interpretation proffered here was further supported by the abolishing of the lag phase during the concurrent application of inhibitor and stimulant, as the microorganism was deemed to be capable of obtaining its supply of acetyl CoA from low- level concentration of 2,4-dichlorophenoxyacetic acid.

E. GENERAL CONCLUSION

The fundamental theory enunciated here was based on the sequence of morphological changes observed in experimental studies, which correlated the growth phases with physiological processes. It was assumed that transport mechanisms were established across the membranes of induced morphologies and this allowed inter-medial fluxes of ions and substrates. Arising from inherent intracellular communication, activated were second messengers generation and protein kinase cascades that gave tenor to DNA replication and, subsequently mitogenesis.

The importance of transmembrane-pH-gradient was emphasized. Unfortunately, in transforming species of this nature, with several transient morphologies, quantitation of transport operations have not been done. It is also probably true of such phenomena with biochemical relationships at the molecular level. However, attempts have been made to give magnitude to ionic movements in such transforming species (*in preparation*). This has led to examination of the effects of critical concentration, chemical potential and Na^+ influx rate on the ability of sporangiospores of *Mucor circinelloides* Tieghe to transform to terminal budding yeast cells. It was further showed that the underlying basis of the transformative process could be anchored on the high cooperativity of these biochemical parameters, including critical concentration, chemical potential, and Na^+ influx rate which physiologically drive the process through specific morphologies, from sporangiospore through protoplast to terminal budding yeast cell.

We have shown in this study that inhibitory substances, as illustrated in fig. 51, did not cause morphogenetic interconversions, although they severely affected cell physiology and proliferation. If the principles outlined here prove to be logical, they may well sustain the basic premise of the SSYT hypothesis and further offer insight into specific phases or reaction steps in the conversion process of a single morphorgenetic species. For instance, Uno *et al.* (1988) developed monoclonal anti body specific for phosphatidylinositol diphosphate and on introduction into yeast cells through electroporation, demonstrated the importance of second messengers in controlling the mitogenic cycle and cell proliferations in *S. cerevisiae.* A new vista could be derived by conducting such experiments through the whole range of morphogenetic transformation in dimorphic microorganisms.

F. SUMMARY

Experimental observations led to the formulation of a hypothesis known as sequential sporangiospore-yeast transformation hypothesis, which holds that mould-yeast conversion occurs in identifiable sequence. This work is a review of relevant experimental background and ends with experimental evidence that tends to give validity to the basic assumptions made therein. It assumes a functional relationship with transductive energy metabolism, fermentative metabolism and cell wall biogenesis. Entreated precepts include the classical Mitchellian chemiosmotic principles, membrane phenomenology and polyelectrolytic mechanism. It further states that yeast cell wall biogenesis is not a consequence of respiratory inhibition but recourse to glucan biosynthetic pathway, which is coupled to fermentative metabolism. The theory advocates a functional relationship through transmembrane-pH-gradient, wall lysis, protoplast formation, second messenger generation, protein kinase cascades and the induction of early genes, which go to specify cellular metabolism and cell wall biogenesis. Evidence is cited in support of the theoretical concepts.

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