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"Development of High Phytase Baker's Yeast"

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The bulk of this work was carried out by the investigator
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"DEVELOPMENT OF HIGH PHYTASE BAKER'S YEAST"

BY GHALEB M. ABUEREISH (& AYED S. AMR)

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Executive Summary

The impetus of this project was to develop a baker's yeast that produces an enzyme which degrades the anti-nutrient substance, phytate, which is present at high levels in the grains and their products. This substance causes deficiency in zinc, calcium and iron at the intestinal level, & their deficiency leads to malnutrition particularly among populations that consume the bread as the main source of energy. One of the project's goals was first to establish whether the baker's yeast is capable of taking up the phytate or not. In this study, we have found that the entry of the phytate into the yeast cells is not a limiting factor in its degradation. The limiting factor, which was the second goal, is that the cells do not produce the correspondent enzyme(s) to degrade it under the ordinary conditions of their growth. After extensive work a strain of the currently used baker's yeast, *Saccharomyces cerevisiae*, has been adopted and selectively isolated. In liquid media, this strain utilized the phytate very well at levels that exceed those present in the dough. Three problems remained to be tested: 1) fermentation of dough by the strain & checking its effect on the phytate level, 2) strain stability under the conditions of doughmaking & 3) defining the enzyme(s) that it produces. However, the strain is useful, because it is suitable for future work such as its genetic manipulation to produce high amounts of the enzyme that degrades the phytate within acceptable time of dough fermentation. This achievement is beneficial for all the world. In addition, our laboratories in biochemistry & nutrition became better equipped. Finally the project has created new avenues & ideas of research which strengthened our graduate and technical training programs.

4) Research Objectives:

Most of the phosphorus in plant seeds is present as phytate, myoinositol hexaphosphate (IP6). [Asada *et al.*, 1969; Erdman, 1979; Oberleas, 1971]. The amount of phytic acid in these products varies from 0.5 to 6% and accounts for 50-90% of the total phosphorus which is not absorbed by the body [Nolan *et al.*, 1987]. In man, dietary phytate reduces the bioavailability of zinc & iron [Atwal *et al.*, 1980; Ferrando, 1987; Hallberg *et al.*, 1989; Harland & Oberleas, 1987; Morris, 1986; Navert *et al.*, 1985; Solomons, 1986; Andersson *et al.*, 1983; Southon *et al.*, 1988; O'Dell & Savage, 1960; Shah *et al.*, 1976]. Phytate forms insoluble complexes in the intestines [Oberleas *et al.*, 1966; Peddy *et al.*, 1982; Maga, 1982]. The effect of IP6 esters on *in vitro* & *in vivo* digestion of proteins has been investigated [Knuckles *et al.*, 1989]. The phytate/nutrient interaction & the effect of phytic acid on nutritional status of humans have been reviewed [Cheryan, 1980; Peddy *et al.*, 1982; Graf, 1986]. Furthermore, the phytate esters are formed during processing [de Boland *et al.*, 1975; Harland & Harland, 1980; Reinhold, 1975; de Lange *et al.*, 1961; Nayini & Markakis, 1983]. The phytate and its esters have been shown to decrease α -amylase

and lipase activities *in vitro* [Sharma *et al.*, 1978; Thompson & Yoon, 1984; Deshpande & Cheryan, 1984; Knuckles & Betschart, 1987; Knuckles, 1988], and inhibit *in vitro* calcification of ricketic rat cartilage and rat aorta [van den Berg *et al.*, 1972]. *In vitro* & *in vivo* studies with phytate hydrolysates showed that myoinositol phosphate esters inhibit peptic and tryptic digestion of some proteins [Knuckles *et al.*, 1985, 1989; Singh & Eriksorian, 1982]. A number of methods have been devised to lower phytate levels or to remove phytate from certain foods [Chang *et al.*, 1977; Harland & Oberleas, 1987; Cosgrove, 1980; Larsson & Sandberg, 1990; Lingaas & Sandberg, 1990; Nasi, 1990; Anno *et al.*, 1985; Bos, 1988; Fretzdorff, 1989]. The effect of fermentation on the level of phytate in cereal products have been investigated [Hetarpaul & Chauhan, 1989; Navert *et al.*, 1985; Nayini & Markakis, 1983; Harland & Frelich, 1989; Bartnik *et al.*, 1987; Pringle & Moran, 1942; de Lange *et al.*, 1961; Reinhold, 1972, 1975; McKenzie-Parnell & Davies, 1986; Mahajan & Chauhan, 1987; Tangkongchitr *et al.*, 1981; Sudarnadji & Marlalis, 1977; Banotra & Loeve, 1975]. In these reports, the figures pertaining to phytate destruction in breadmaking vary from 100% for white bread [Pringle & Moran, 1942], to 72-77% for whole wheat bread & 70% - extraction wheat bread [Nayini & Marlalis, 1983], to 40 - 50% for whole wheat bread [de Lange *et al.*, 1961], to 13% for

village flat breads made in Iran [Reinhold, 1972] and to 0-20% for 100% - extraction wheat bread [Harland & Frolich, 1989] after two hours fermentation. The reports attributed the reduction of phytate in the bread to the activity of the enzyme phytase (EC 3.13.8 & 3.1.3.26, IUB, 1979). However, the reports on phytase activity in the yeast are in conflict. Nayini & Markakis, 1984, had partially purified an enzyme from yeast extract which hydrolyzes IP6 and other compounds of organophosphate. In this report the phytase activity was minor compared to that activity towards pyrophosphate (1/371) or p-nitrophenyl phosphate (1/40). The optimal pH, temperature, & substrate concentration for this enzyme were 4.6 & 45°C, & 1.0 mM, respectively. The Km was 0.21 mM; and 85% inhibition by the substrate was at 6 mM of IP6. Other reports support that the yeast enzyme is nonspecific acid phosphatase [Bartnik *et al.*, 1987; Barbaric' *et al.*, 1980, 1984; Lingaas & Sandberg, 1990] or pyrophosphatase [Heppel, 1955]. Furthermore, most of the reports on doughmaking and phytate degradation by yeast phytase lacked several important factors. Of these are a control experiment in which only the wheat flour is included (i.e. no yeast is added to the dough), the effect of inorganic phosphate on the breakdown of phytate, and the effect of phytate on the yeast growth. The presence of phytase in the wheat was reported [Peers, 1953; Nagai & Funahashi, 1962, 1963]. Nagai & Funahashi (1962)

concluded that the enzyme was nonspecific acid phosphatase. To our best of knowledge, the wheat and yeast enzymes have not been well documented. Recently, however, phytic acid has been shown to have antioxidant activity when complexed with ferric ion. The level of phytate that is needed to this activity is much less than that is present in foods (i.e. μM vs. mM ; Empson *et al.*, 1991). Another aspect of the problem of phytate is that no reports have been published on the level of phytate that would bind zinc, iron, and calcium in the intestine or at least *in vitro* studies.

In order to improve the quality of bread and other wheat products, several criteria have to prevail. The flavor, the nutritional quality, and the bioavailability of the minerals: zinc, iron, and calcium. The flavor is related to the time of fermentation, and the minerals bioavailability is related to phytic acid content in the dough. This study has dealt with the ways of improvement of the bioavailability of the minerals by finding ways of elimination of phytic acid from the dough through fermentation process which should be within reasonable and relatively short time in order to preserve the flavor of the bread. Therefore, the aims of this study were to survey the phytase activity in a number of yeast isolates from various food sources, to investigate the effect of phytate on yeast growth in liquid media in presence and absence of inorganic phosphate, to study the phytate transpor-

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rt into the yeast *Saccharomyces cerevisiae*, to examine the effect of various carbon sources on the utilization of phytate by the yeast *S. cerevisiae* and to isolate a strain that is capable of production of phytase in response to phytate under specified conditions such as lack of phosphate source and synthetic media.

5) Methods and Results

Methods

Materials- Baker's yeast was obtained from local bakeries and from and from the local supermarkets. Noncommercial *Saccharomyces cerevisiae* and other yeast isolates were isolated in this laboratory. Phytic acid and ampicillin were from Sigma Chemical Co. Saint Louis, MO. USA. The media for yeast cultures were purchased as following: Potato dextrose agar, malt agar, M. standard methods broth & M. trypticase - glucose- yeast broth, which were used for yeast cell counts & for yeast isolation, were from DFL; peptone and yeast extract, which were used at early stages of this study for yeast culturing in liquid media, were from HI MEDIA LABS. Pvt. Limited. Bombay, India; yeast nitrogen base (YNB), bacto-agar, yeast extract, bacto-peptone, glucose, and sucrose, which were used later on the study, were from Difco

Labs., Detroit, Michigan, USA. All other chemical reagents were analytical grade. Distilled water was used through out.

Procedures- Cultures of yeast cells- the cells were grown aerobically on agar plates or on various media as specified in their corresponding experiments according to Schatz, 1979; Leininger, 1976; Schweingruber & Schweingruber, 1979; Barbaric *et al.*, 1980; Elorza *et al.*, 1978. Phytic acid, glucose, sucrose, and ampicillin were sterilized by millipore filtration (Sartorius, Germany). Other media were sterilized in an autoclave (Scientific Precision, model 8000-DSE) at 121°C, and 15 psi for 20 min.

Identification of yeast isolates was according to Deak & Beuchat, 1987.

Preparation of inorganic phosphate-free media was according to Rubin, 1973.

Yeast growth was determined by cell counts (i.e. serial dilution, plating on agar plates and counting colonies or based on by measurements of turbidity at 550 nm using Pye Unicam or Perkin-Elmer spectrophotometer (Rubin, 1973). At absorbance of 1.5 was found to correspond to 10^7 cells/ml. Specific growth rate and doubling time were estimated according to Rose & Harrison, 1987. Purity of yeast cultures was examined by staining and microscopic examination.

Separation of cells from liquid media was through

centrifugation in an Eppendorf, refrigerated microcentrifuge. Dough formulation- 100 g of whole wheat flour (72 % extraction) (WWF) were mixed with, 1.5 g salt, 5.0 g sucrose and 70 ml water. To 8.5 g of the mixture, 1.0 ml of yeast suspension (1.0 g of yeast plus 5 ml water) was added. A control sample was taken to which 1 ml of water was added. The dough was incubated at 32°C for specified period, dried, and analysis for phytate was carried out.

Phytic acid determination- phytic acid was determined according to the method of Wheeler and Ferrel, 1971, and to the method of Latta & Eskin, 1980, as modified by Vaintraub Lapteva, 1988. Determination of inorganic phosphate- free phosphate in the media was determined colorimetric (Gibson & Ullah, 1988). Yeast extracts preparation- The method of Schatz, 1979, was used. Glass beads (40 mesh) were treated with 5N HCl solution & rinsed with distilled water. The yeast cells were suspended in a least volume of water (or buffer) & the glass beads were added to the suspension at 3.5:1 (v/v), respectively, as vortexing the mixture in a small test tube (2x20 cm) for 5 min or more if necessary. For large quantity of cells, the cells-glass beads mixture was agitated by magnetic stirrer for the period required to break the cells as the breakage monitored by microscopic examination. Enzymic assay for phytase and pyrophosphatase was performed as described. WWF and bran extracts- The

method of Peers, 1953, was followed. Six parts of flour or bran were extracted with 35 parts of water for 6 h at 0 - 4°C (pH 7.6), followed by centrifugation at 20,000 x g for 20 min to obtain clear supernatant which is assayed for phytase activity as described below.

Enzymic assays- The strategy of measuring the activity of the enzyme(s) that hydrolyze(s) inositolhexaphosphate (phytate, IP6) or inorganic pyrophosphate (PP_i) is based on the determination of inorganic phosphate (P_i) released from the substrate used in the assay.

Phytase assay- The method of Gibson & Ullah, 1988, was slightly modified and used to assay for yeast enzyme. Each assay contained 37.5 µmoles of Na acetate buffer, pH 4.6 & 0.5 µmole of Na-IP6 (or Na pyrophosphate).

reaction was started by the addition of the yeast extract. The final volume of the reaction mixture was 1.0 ml. The incubation was carried out at 45°C for one hour. To each assay, 2 ml of freshly prepared mixture of 2 volumes of acetone, 1 volume of 5 N H₂SO₄ solution & 1 volume of 10 mM ammonium molybdate solution were added. The absorbance of the yellow color was measured at 355 nm or 410 nm. The pH and temperature were taken according to Nayini & Markakis, 1984. Blanks, controls and standards were also prepared in the same manner. A standard curve for P_i ranging from 2.5 nmoles to 200 nmoles was established. The absorbance for 10

nmoles was around 0.025 at 355 nm. A unit of phytase activity under the conditions of the assay (Nayini & Markakis, 1984) is defined as the amount of the enzyme in the extract from 0.15 g of dry yeast cells that liberates one μ mole of P_i per min.

For WWF & bran phytase, the method of Peers, 1953, was used. The assay mixture contained 0.1 M Na acetate buffer, 4 mM $MgSO_4$, the extract & 1.6 mM Na phytate in a total volume of 1.2 ml, pH 5.15. The reaction was started by the addition of phytate and was continued for one hour at 55°C. An aliquot was withdrawn and added to one half of its volume of 10% TCA solution. The precipitate was removed by centrifugation, and the P_i was determined as described above. A unit of phytase activity under these conditions is conditions as the amount of enzyme in the extract from one mg dry material, that liberates one μ g phosphorus per one hour. (This definition was taken for comparing the results obtained in this report with those of Peers, 1953). Transport of phytic acid in yeast cells- the cells were suspended in a buffer containing 1 mM phytate and were incubated at 28°C in rotary shaking water bath. At specified time intervals, aliquots were removed, cells were separated by centrifugation and exogenous phytate inorganic phosphate were determined in the medium. Effect of pH on the phytic acid degradation by WWF enzyme(s)- 100 g of WWF, 72% extraction, were mixed with 70 ml of HCl

solution that is required to drop the pH from 6.21 (the actual pH of the mixture) to 5.15 (the optimal pH of the wheat phytase). A control sample was mixed with 70 ml of water (pH 6.21). At time of intervals, 8.5 g samples from each of the above were taken and used for phytate assay.

RESULTS AND DISCUSSION

The activity of phytase in the extracts of baker's yeast cells ranged from 12.6 nmoles to 28.0 nmoles P_i /min /0.15 g dry cells. The pyrophosphatase activity in the same extracts was 405 nmoles P_i /min / 0.15g dry cells. The pyrophosphatase activity was fully inhibited at 9 mM Ca^{++} ; while the phytase activity was not affected. The phytase activity here confirms the findings that reported by Nayini & Markakis, 1984; and the inhibition of pyrophosphatase by Ca^{++} confirms the results of Heppel, 1955. Furthermore, the results of inhibition by Ca^{++} in this report indicate that the phytase is distinct from pyrophosphatase. The assay for pyrophosphatase was performed in order to confirm this point, and check the method for the assay of phytase and to insure that yeast cells are adequately broken. The method of assay for the pyrophosphatase is different from what is performed in this study, and the actual activity by that method is 17 μ moles P_i /min/0.15g dry cells. These results suggest that the phytase

activity is due to a nonspecific acid phosphatase or it is due to a mixture of phosphatases and pyrophosphatase. The phytase activity in the extracts of WWF and bran are presented in table 1. The results indicate that WWF and its bran contain phytase activity. The phytase activities in our samples are higher than those obtained by Peers, 1950; but the ratio of the enzyme activities in the flour and in the bran is essentially the same, 0.79 *versus* 0.65, respectively. The rate of disappearance of exogenous phytic acid (IP6) from the media in the presence of yeast cells, *S. cerevisias*, and the rate of the simultaneous appearance of inorganic phosphate in the media at 1.0 mM IP6 without glucose, any other carbon source, or exogenous phosphate are shown in Figure 1. The data show that as IP6 level decreases P_i level increases in the medium. Within 75 min., both levels reach their end points and remain so for 150 min of incubation. Also in the figure, the rate of disappearance of IP6 is shown. Figure 2 represents the rates of degradation of IP6 at various concentrations, and production of P_i by yeast cells. Two control experiments were used: one control contains phytic acid and the yeast cells; the other control experiment contains phytic acid. From each experiment, aliquots were removed at time intervals and analysis for IP6 and P_i in the cell-free supernatant were performed. The results in the figure indicate that initial IP6 uptake

reached 0.7 to 0.8 mM (in both controls at exogenous IP6 of 3.0 to 4.0 mM). The P_i level, however, reached maximum value of ~ 2.5 mM in the medium at 1.0 mM IP6. P_i in the phytic acid solution was less than 0.08 mM which agreed well with that indicated by the manufacturer ($\sim 0.1\%$ P). The apparent formed extracellular P_i declined to less than 0.4 mM at IP6 of 8 mM. In the meanwhile, the uptake of IP6 is presented in two curves in this figure. In curve A the control was IP6 without cells, and in curve C, the control was cells plus IP6, but no incubation. In both curves IP6 consumption reached maximal at 3 mM then declined to 0 mM and ~ 0.3 mM in curves A and C, respectively. IP6 consumption remained so in curve C, but increased again in curve A. The difference in the two patterns might be due to metabolic activity and to different phosphatases. In curve C the enzyme phytase is inhibited at 5 mM and this confirms the results obtained by Nayini and Markakis, 1984. The increase in IP6 consumption at higher IP6 concentrations could be explained by the cells activity at 28°C and possibly by the action of other phosphatase(s). In attempt to find a suitable yeast strain that degrades IP6 at higher rates, a number of isolates were identified and examined. Table 2 represents the source and identification of each yeast isolate and its action on IP6 in the WWF dough. An interesting (if not puzzling) finding is that the control (flour mix that did not have yeast) gave a

better activity (0.213 g %) than those which have yeast (0.364 to 0.962 g %, see those marked with †). Three yeast species, *P. guilliermondii*, *R. mucilaginosa* and *S. cerevisiae* reduced IP6 level to 0.121 to 0.197 g %. These findings indicated that the flour contains either enzyme(s) or certain microorganisms that affect the IP6 level which is at least 0.96 g %. To account for the contradictory results, it was thought that IP6 might not be distributed evenly in the flour which was used in dough making. Also the time and the temperature of the drying process of the dough making were not well defined. Therefore, it was thought that the process must be repeated under controlled conditions. The same experiments were repeated, but a quantity of flour (100 g) was mixed with water (70 ml). A quantity of the mixture (8.5 g) was taken to which added 1.0 ml of yeast suspension (made as 1.0 g yeast plus 5 ml water). The dough was then incubated at 32°C for 2 h, dried and then analyzes for IP6. Table 3 shows the results of such experiments, which point out: 1- The levels of IP6 in the doughs which had boiled yeast suspension are much higher than those were obtained from control experiments in table 2 (i.e. 0.617 - 0.741 v.s. 0.213 g %), respectively. 2- Apparently, isolate # 10 (*S. cerevisiae*) is more sensitive to heat than the other isolates because it is the only one which did affect the phytic acid level in the control experiments after 2 h incubation (0.617

v.s. 0.733 g %). 3- In the experimental column, some doughs show significant decrease in the level of IP6 even they were not incubated at 32°C, which might be attributed to lengthy time of drying. 4- Inconsistent results among the repeated experiments which dealt with the same yeast (i.e. compare values in table 2 with the values in the last column in table 3 for isolates numbered 6, 7 & 11). Most of the previous reports which have been published on the effect of yeast on the levels of IP6 in the WWF dough failed to include control experiments (i.e. without yeast as in Navert *et al.*, 1985). Table 4 includes the levels of IP6 in WWF doughs during their incubation at 32°C for various periods (no yeast was added). In all experiments, 77 % of the initial IP6 was destroyed (0.890 versus 0.205 g %). Qualitatively, these results are in concordance with those found by Lingaas & Sandberg, 1990, in that endogenous phytase contributes to the reduction of phytate in the dough; but their results showed 64 % reduction after two hours of fermentation. The effect of pH of the dough was also studied, and is presented in figure 4. Dropping the pH to phytase optimal pH for activity did not significantly lower the level of IP6 faster than that at pH 6.12, the ordinary pH value of the dough. The difference in the patterns of the curves might be due to the difference in the initial IP6 contents in the doughs although the samples were taken from the same flour mixture. This discrepancy,

however, is common in this type of work and cannot be explained at this stage. Apparently, initial incubation of the dough mixture without the yeast at least for 20 min followed by the addition of the yeast may contribute to further degradation of the IP6 by the yeast and the wheat enzyme. Locally there are two commercial baker's *S. cerevisiae*. The Astrico, dry pelleted yeast which is sold in the supermarkets and the compressed yeast which is used in the bakeries. Figures 4-5 represent the activities of the two types of yeast towards the utilization of IP6. Slight difference between the two types is observed at early times of incubation, however, the uptake of IP6 by both is essentially the same at two hours of incubation as indicated by the release of P_i , 6 mM, at 1 mM IP6. Most important is the inhibition of P_i release as [IP6] increases. Apparently the Astrico type is less sensitive to IP6 than the compressed yeast. The experiments were repeated on representative previous isolates, and typical results are shown in figure 6. None of these yeast isolates did show the rate of IP6 uptake, which was obtained from the commercial *S. cerevisiae*. However, *S. cerevisiae* from labaneh (concentrated yoghurt) gave better activity than the others. These results led to the thinking that the commercial *S. cerevisiae* might have strain(s) that could adapt to IP6. Therefore, compressed *S. cerevisiae* which grew in 5 mM IP6 in liquid media, was plated

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on agar containing IP6, pH 3.2. After 10 days at 28°C, clear colonies appeared as shown in plate 1. One of the colonies had an orange to yellowish color with tri-dentate (deltoid) morphology. This colony was designated Y-5-a. Another colony which had the crescent shape (concave) with orange color was designated Y-5-c. Spherical colonies were shown to be bacteria and had no IP6 uptake activity.

Before challenging the cells of colonies above with higher IP6 concentrations, the effects of carbon source and ethanol on the cell growth and IP6 uptake were examined. Table 5 shows the results of the study of the effects of carbon source and ethanol on the growth of *S. cerevisiae* and the utilization of IP6 by the cells.

The results indicate 1- Low [IP6], i.e. > 2 mM, inhibits the enzyme(s) that degrade(s) it, but the IP6 level does not inhibit the yeast growth. This result confirms the findings that are presented in the figures 1-2. 2- Subculturing of *S. cerevisiae* cells that were grown on low IP6 levels in media that contain higher IP6 levels resulted in the adaptation of the cells to the high levels of IP6 and its degradation, i.e. compare results of cultures grown on 8% sucrose + 5 mM IP6 to those grown on 8% sucrose + 5 mM IP6 (in the table). 3-As IP6 increases its uptake and the growth of the cells decrease (see results in experiments that contained 8% sucrose^b and various concentrations of IP6 ranging from 5 mM to 40 mM).

4- Glucose was less effective towards degradation of IP6 than that of sucrose. 5- Glycerol and ethanol did not stimulate cell growth. 6- Ethanol had no effect on the IP6 utilization or on cell growth.

The next experiments were performed on cells from colonies Y-5-a & Y-5-c. Figure 7 represents the rates of cell growth as measured at 550 nm and the media was peptone, yeast extract, 8 % sucrose (4.5 mM endogenous P_i), 40 mM IP6, and 100 μ g ampicillin/ml. The cells in this experiment were previously cultured in liquid medium containing 5 mM IP6. The figure indicates that IP6 at this level increased the doubling time of both types of cells. However, apparently Y-5-a has less doubling time of growth than Y-5-c. Therefore, for this reason, the next experiments were performed on Y-5-a. Figures 8-10 show the pattern of growth of Y-5-a in yeast nitrogen base - 8% sucrose media containing various IP6 concentrations plus other additives as indicated in the figures. Figure 8 shows that as IP6 level increases from 10 mM to 30 mM the growth rate decreases. Figure 9 & 10 shows the same type of experiments with IP6 levels at 30 & 40 mM, and the effect of exogenous inorganic phosphate at 50 mM. Two significant observations may be concluded from the two figures: 40 mM IP6 delayed cell growth for a long period, and inorganic phosphate, some how, partially relieved the inhibition. Also the P_i effect indicates that the enzymes

induced are not acid phosphatases because these would be inhibited by high P_i (Mildner *et al.*, 1972; Elorza, *et al.*, 1978; Schwergruber & Schwergruber, 1979). The specific growth rate and the doubling time were estimated from the plot of $\ln E$ at 550 nm (where E represents the cell mass) versus time and the equation $T_d = 0.693/\text{specific growth rate}$, where the specific growth rate is the slope of the straight line in the graph. A typical graph is presented in figure 11. The doubling time of the cells is computed at 5.54 h. When the cells were grown on low IPG and then were subcultured in media with higher levels, the cells became adapted to the high levels, and the doubling time became shorter until it reaches the normal doubling time, about two hours (Zinker & Warner, 1976). For instance, when cells, which were taken from culture grown in medium at 30 mM IPG, and inoculated into media of YNB +8% sucrose containing 10, 20, & 30 mM IPG, the doubling times were found as 2.2, 2.4, and 2.7 h, respectively (figure is not shown).

The growth of cells in liquid media containing IPG is accompanied by the release of P_i into the extracellular fluid & the disappearance of IPG from the media. The rates of these parameters were found variable with respect to the type of media. Figures 12 & 13 illustrate this phenomenon. Figure 12 shows that Y-5-a utilizes IPG from YNB medium better than that from FYE medium. Figure 13 shows that P_i release and

IPG uptake are higher at 10 mM IPG than at 20 mM IPG in FYE media which confirm the similar previous findings as YNB media were used. The purity of yeast cultures in the above experiments was confirmed by staining and light microscopy. Typical cultures of Y-5-a are presented in plates 2 & 3 in which living & stained cells were used. The cells were active in division as buds are so numerous. No bacterial cells were observed in the cultures from which the results of this study were obtained. Any culture which showed contamination was discarded. The number of cells in the culture always corresponded to the light scattering obtained at 550 nm, i.e. 1.5 corresponded to 1×10^7 cells/ml (Rubin, 1973).

CONCLUSION

This study established: 1) Under specified conditions *S. cerevisiae* would produce enzymes that degrade phytate. 2) In the dough, as is in current methods of breadmaking, enzymes of the yeast have minor role in utilization of the phytate. 3) The wheat phytase in the dough in absence of yeast would degrade 77 % of the endogenous phytate within 20 to 30 minutes at 32°C, but this level is not known whether it affects minerals absorption in human intestine, and this reduction of phytic acid by endogenous phytase activity was

inconsistent. The variation could be due the age of the flour, temperature of milling, moisture, temperature of storage and so on. 4) The uptake of phytate by the ordinary baker's yeast cells depends on its concentration. At 1 mM of phytate the cells take it up and hydrolyze it by the enzyme(s) as concluded from transport experiments (no cell growth under those experimental conditions). As the IP6 concentration increases to \approx 2 mM the hydrolysis of the compound is inhibited. 5) *S. cerevisiae* strain (Y-5-a) was selected from yeast cells which were allowed to grow first in medium containing 5 mM phytate, then plating the culture on petri dishes containing agar, medium and phytate at pH 3.2. 6) Y-5-a strain grows in high levels of phytate, but this inhibits cell growth at 1-40 mM. This inhibition reflects an increase in cell doubling time accompanied by a lag phase. This phenomenon indicates that this is an adaptation process to this level of phytate by synthesizing enzyme(s) that is/are capable of degrading the phytate and less sensitive to those which were inhibited at lower levels (enzyme induction followed by inhibition). 7) Inorganic and organic phosphates in the media are preferred as source of phosphate for growth. 8) Inorganic phosphate in the medium partially relieves the inhibition by phytate, which suggests that phytate and phosphate transport may share common mechanism and that the enzymes induced may not be acid

phosphatases. 9) No effect for ethanol on the growth of the cells, and sucrose served better as carbon source than glucose. 10) Y-5-a *S. cerevisiae* strain could be an ideal system for cloning and/or amplification of phytate-specific phytase.

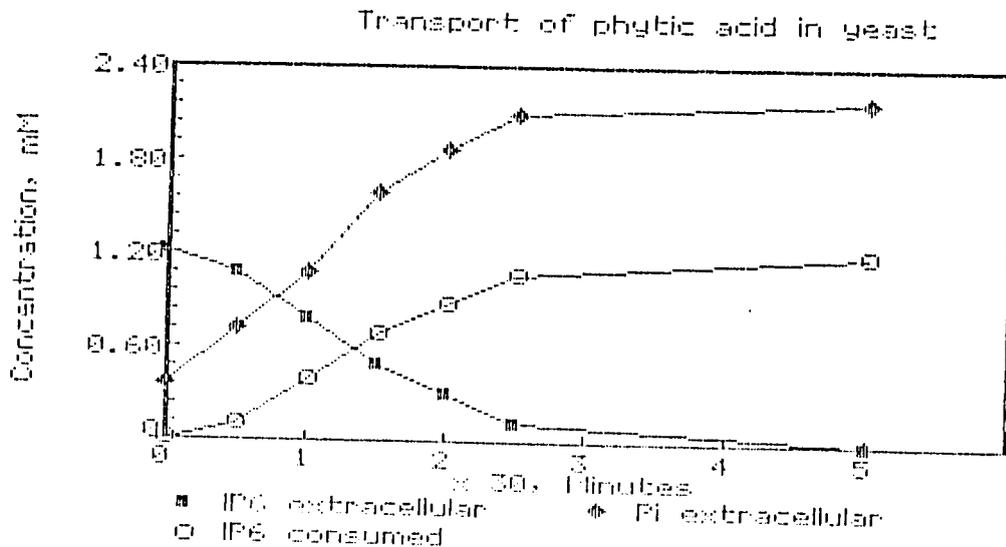


Fig. 1. Transport of phytic acid in baker's yeast. Yeast cells, *Saccharomyces cerevisiae*, were incubated in medium which contained 1.0 mM phytic acid, at 28°C. After 15 min intervals, aliquots were removed, centrifuged to separate cells, and the supernatants were assayed for inorganic phosphate, P_i , and phytic acid was described in methods. Curve C represents the rate of consumption of phytic acid which is computed as the difference between initial phytic acid concentration and the residual phytic acid in medium at the specified intervals.

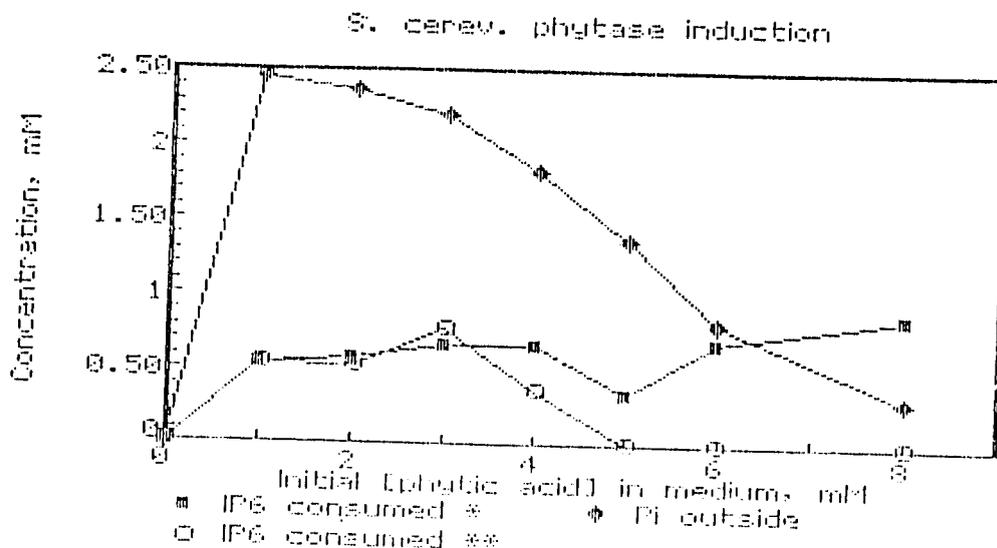


Fig. 2. *Saccharomyces cerevisiae* phytase induction by phytic acid (IP6). The yeast cells were washed twice and suspended in IP6 solution at the specified concentration. One batch of cell suspension was analyzed immediately for extracellular IP6 (control), and another batch was incubated at 28°C for 75 min (test), then extracellular IP6 was determined according to the method described in text. The cells were separated by centrifugation. * Data obtained from test experiment; and the control was phytic acid without yeast. ** data were obtained from test experiment; the control was phytic acid plus cells, but no incubation.

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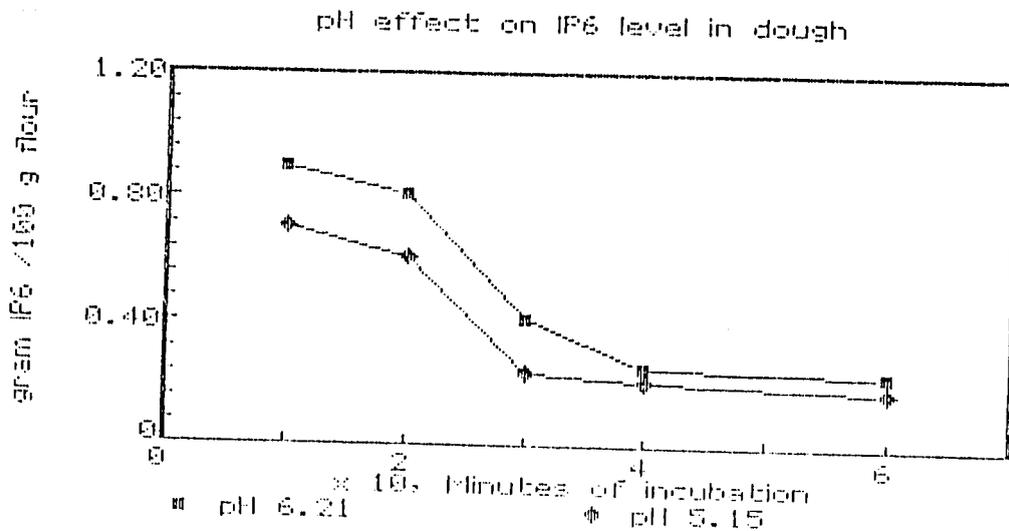


Fig. 3. Effect of pH on the breakdown of phytic acid by the endogenous phytase in the whole wheat flour. One hundred grams of the flour (72 % extraction) were mixed with 70 ml water. The pH of this mixture was measured and found 6.21. Another 100 g were mixed with 70 ml H₂O which contained HCl to bring the pH of the mixture to 5.15. The mixtures were incubated at 32°C for the time intervals as indicated on the graph. Samples, 0.85 g each, were taken at each time interval from each mixture and assayed for phytic acid as described in methods.

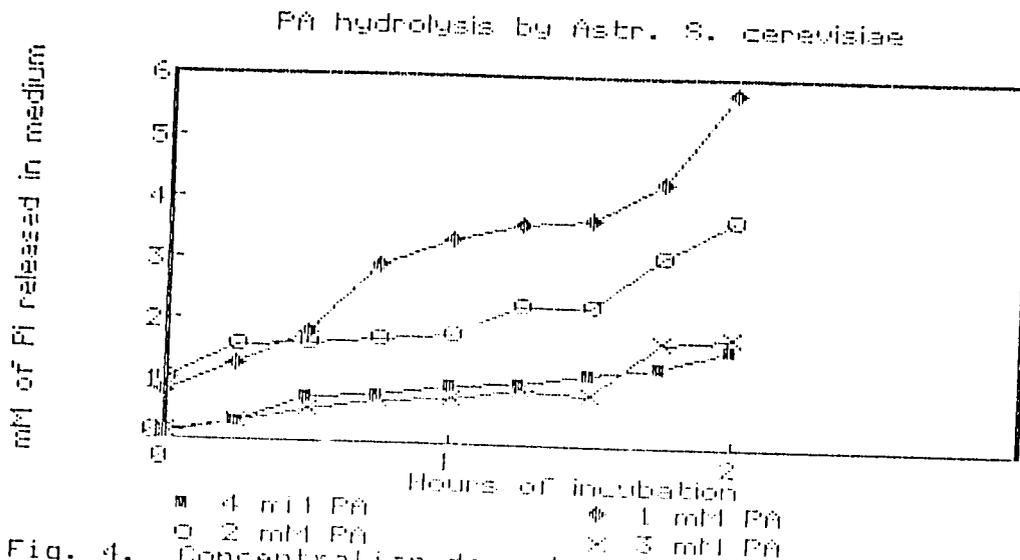


Fig. 4. Concentration-dependence hydrolysis of IPG by *Astrico S. cerevisiae*. Wet cells (1 g) were suspended in 3 ml of medium which consisted of 1 mM glucose, a mM NH_4Cl , 0.85 g $\text{NaCl}/100$ ml, & IPG as indicated, pH 5.2. The cell suspension was incubated at 28°C in a water bath shaker at 200 rpm. After specified time intervals, aliquots were removed and analyzed for P_i in the cell-free medium.

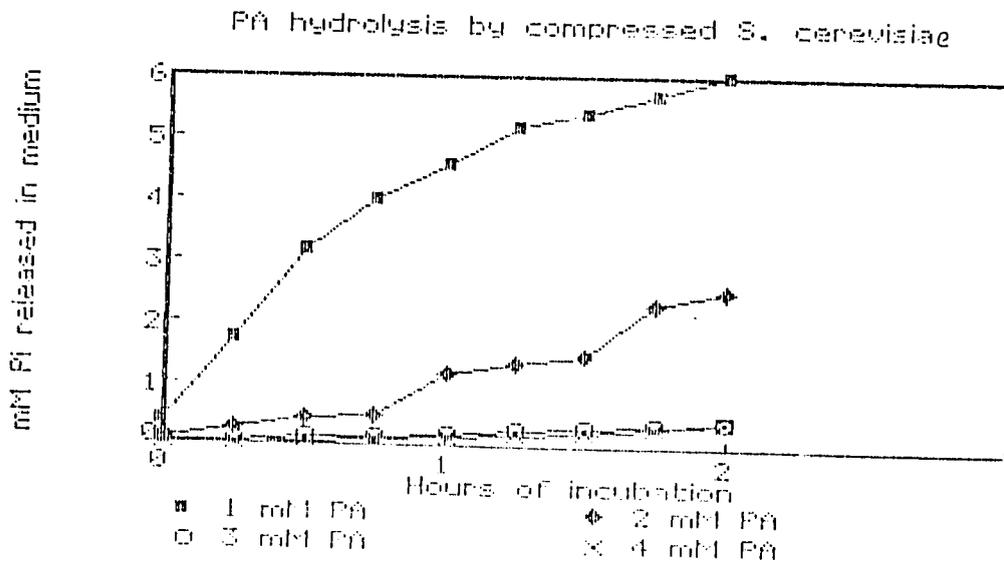


Fig. 5. Concentration-dependence hydrolysis of IP₆ by compressed *S. cerevisiae*. Experimental conditions were as those of figure 4.

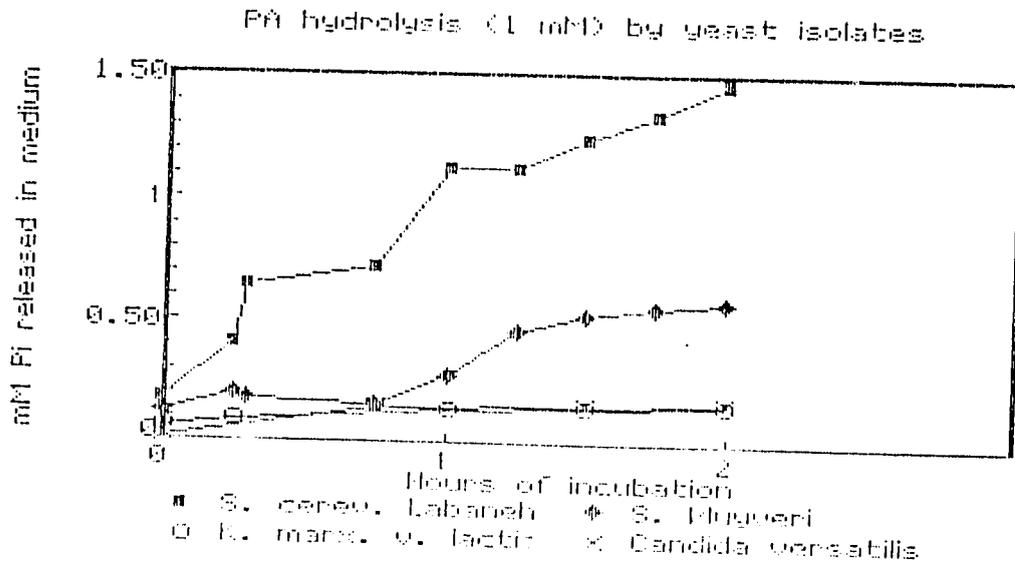


Fig. 6. Hydrolysis of IPG by various yeast isolates. Experimental conditions were as those of figure 4. IPG was 1 mM. The curves refer to, from most upper to lowest, *S. cerevisiae* from labanah, *S. kluyveri*, *K. marxianus var lactis* and *Candida versatilis*, respectively.

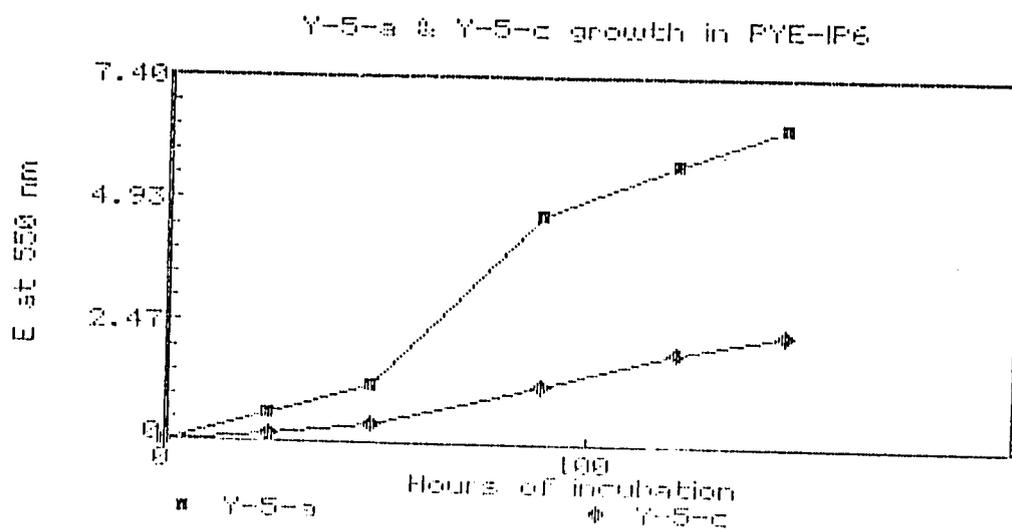


Fig. 7. Growth of *S. cerevisiae* strains Y-5-a and Y-5-c in PYE-IP6 media. The cells from colonies Y-5-a and Y-5-c which were obtained from agar plates that were inoculated with *S. cerevisiae* cells that were adapted to 5 mM IP6. The medium contained PYE, 8% sucrose, 40 mM IP6 and 100 μ g amp/ml. Endogenous P_i was 4.5 mM. Total volume of medium in 250-ml-side-armed flask was 30 ml.

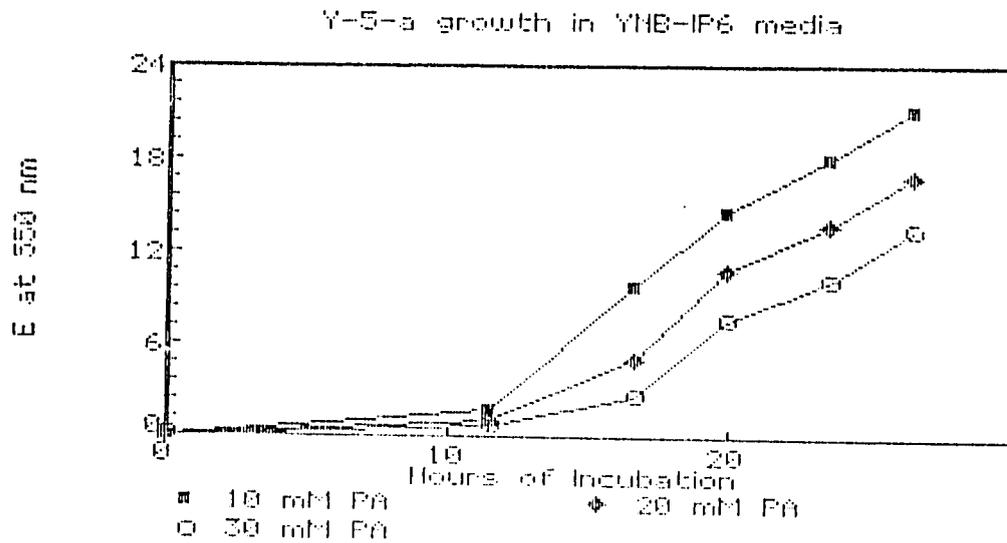


Fig. 8. Growth of Y-5-a in yeast nitrogen base (YNB) medium containing 8 % sucrose and various concentrations of IP6 and 260 μg amp/ml. The inoculum was taken from the same Y-5-a culture previously grown on 30 mM IP6 in YNB - 8 % sucrose - 260 μg amp/ml. Endogenous F_1 in the medium was 6.8 mM.

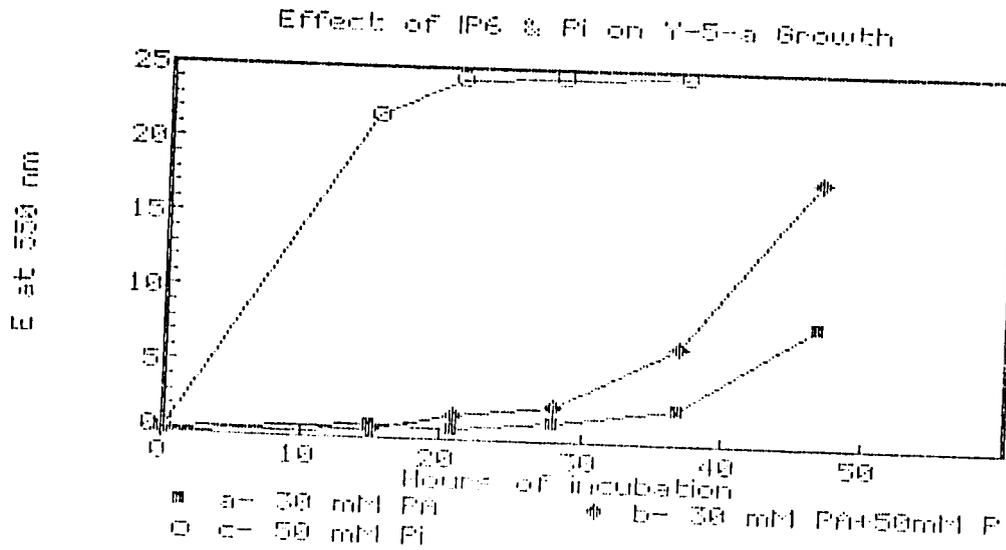


Fig. 9. Effect of 30 mM IP6 and 50 mM P_i on the growth of Y-5-a cells that were adapted to 20 mM IP6. Cells (2x10⁶/ml) were inoculated in YNB medium containing 8% sucrose, 30 mM IP6 and 260 µg amp/ml in presence or absence of 50 mM P_i. Total volume in 250 ml-side-armed flask was 30 ml.

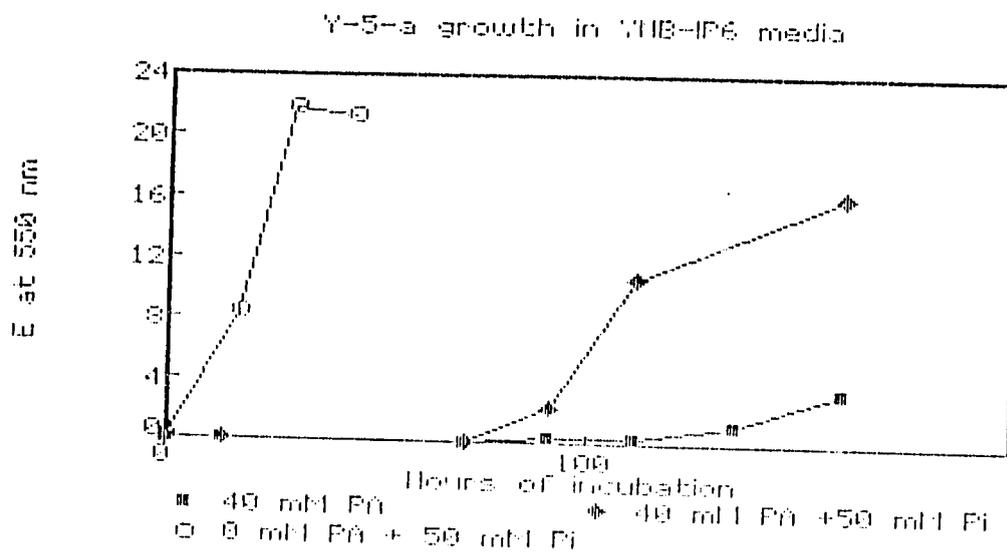


Fig. 10. Effect of 40 mM IP6 and 50 mM P_i on the growth of Y-5-a cells that were adapted to 20 mM IP6. Cells (2×10^6 /ml) were inoculated in YNB medium containing 8% sucrose, 40 mM IP6 and 350 μ g amp/ml plus or minus 50 mM P_i . Total volume in 250 ml-side-armed flask was 30 ml.

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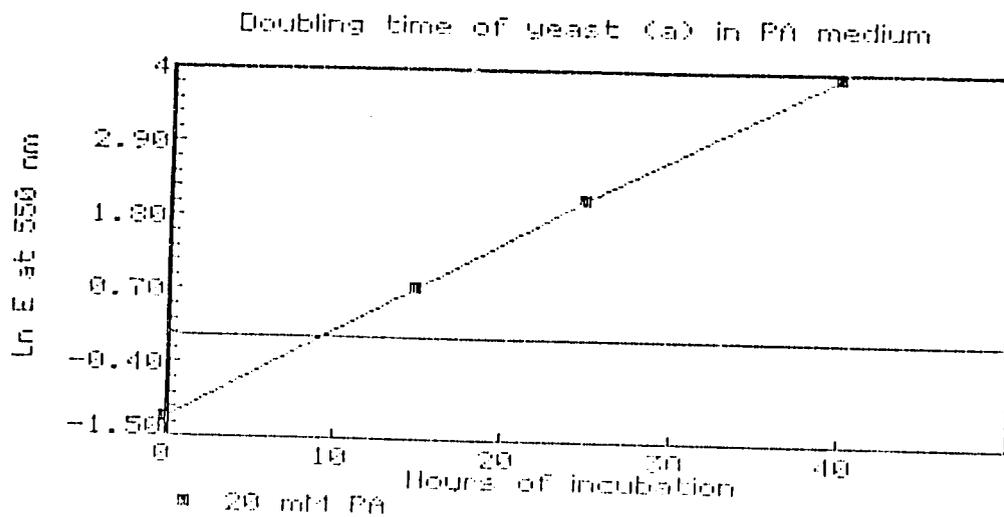


Fig. 11. Doubling time (t_d) of Y-5-a which was adapted to 5 mM IP6 in liquid medium before inoculation into YNB - 8 % sucrose medium containing 20 mM IP6 & 200 μ g amp/ml. The inoculum was 2×10^6 cells/ml, and the total volume was 30 ml in 250 ml side-armed flask.

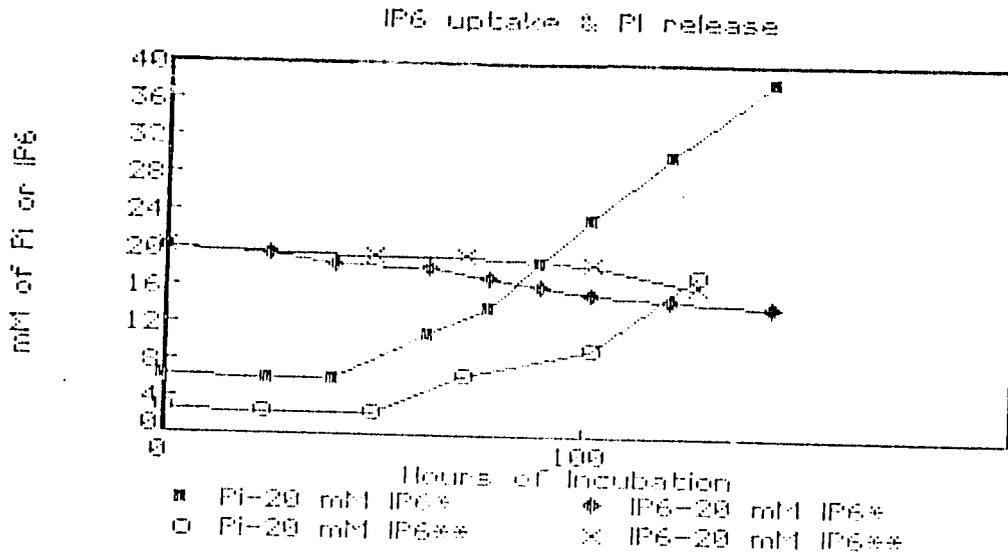


Fig. 12. Effect of media composition on the IPG uptake by Y-5-a and the release of P_i. Cells were inoculated at 2 x10⁸/ml in YNB or PYE media containing 8 % sucrose, 20 mM IPG and 260 µg amp/ml. Total volume was 30 ml in 250-ml side-armed flask. Profiles in squares were obtained from YNB medium and the others from YPE medium. * P_i released from 20 mM IPG in YNB medium. ** P_i released from 20 mM IPG in PYE medium.

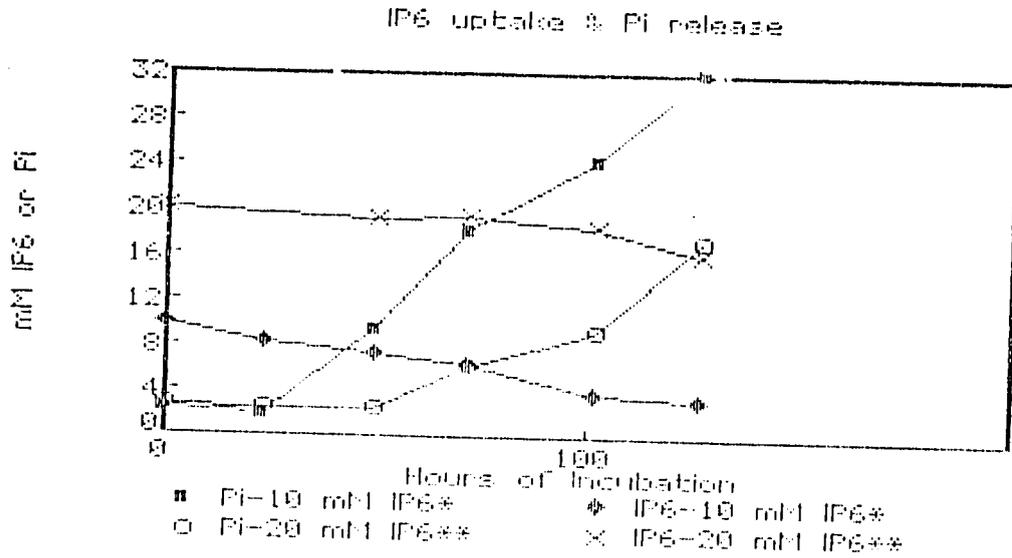


Fig. 13. The effect of IP6 concentration on its uptake and on the release of P_i by Y-5-a inoculated at 2×10^6 /ml in PYE medium containing 8% sucrose and 260 μ g amp/ml. Total volume was 30 ml in 250-ml side-armed flask. ¶ Profiles in squares represent the P_i released from IP6 at 10 mM initial concentration. ¶¶ Other profiles represent P_i released from IP6 at 20 mM initial concentration.

Table 1- Phytase activity in the extracts of whole wheat and its bran, and comparison with Peers data.

	Phytase activity†, $\mu\text{g P}_i$ /h /mg dry weight		
	Flour	Bran	Flour/Bran ratio
This Study	5.08	7.90	0.65
Peers, 1953	3.41	4.34	0.79

* For the assay of phytase activity, see text.

Table 2- The effect of yeast isolates from various food sources on the level of phytic acid in the whole wheat flour after incubation for 2 h followed by drying.

Source	Yeast isolate	Gram of phytic acid per 100 g flour
Control	No yeast added	0.213
Plum jam	1. <i>Candida glabrosa</i> *	0.375 ± 0.037
Olives	2. <i>Candida etchellsii</i> *	0.855 ± 0.010
Apple jam	3. <i>Candida versatilis</i> *	0.712 ± 0.062
Fresh grape juice	4. <i>Hsp. osmophila</i> *	0.598 ± 0.027
Yoghurt	5. <i>Kluyveromyces marxianus var lactis</i> *	0.962
Apricot juice	6. <i>Metschnikowia pulcherrima</i> *	0.364
Mandarin juice	7. <i>Pichia guilliermondii</i>	0.160 ± 0.009
Grapes, dried	8. <i>Pichia membranaefaciens</i> *	0.397
Orange juice	9. <i>Rhodotorula mucilaginosa</i>	0.197 ± 0.010
Dough	10. <i>Saccharomyces cerevisiae</i>	0.121 ± 0.009
Grape juice fermented for 72 h	11. <i>S. cerevisiae</i>	0.121 ± 0.009
Labaneh	12. <i>S. cerevisiae</i>	0.211
Orange juice	13. <i>S. Kluyveri</i> *	0.927
Mandarin juice	14. <i>Sterigmatomyces nectairii</i> *	0.817 ± 0.002

Table 3- Effects of yeasts numbered 6, 7, 9, 10 and 11 (in Table 2) on the levels of phytic acid in homogenized whole flour dough.

Yeast number and name	Grams of phytic acid / 100 g flour			
	Control-a		Experimental	
	0 h	2 h	0 h	2 h
6. <i>M. pulcherrima</i>	0.715	0.307	0.161	0.140
7. <i>P. guilliermodii</i>	0.705	0.275	0.810	0.528
9. <i>R. mucilaginoso</i>	0.741	0.278	0.204	0.162
10. <i>S. cerevisiae</i>	0.617	0.733	0.621	0.196
11. <i>S. cerevisiae</i>	0.735	0.288	0.723	0.388

a- Yeast suspension was boiled five minutes before its addition to flour mixture. Controls and experimental doughs were incubated at 32°C for the time periods as indicated before drying and determination of phytic acid.

Table 4- Levels of phytic acid in whole flour dough *versus* time. One hundred grams of flour were mixed with 70 ml of water (pH measured at 6.21). Eight grams of the mixture were taken for each experiment.

Incubation period, hours	N	Grams of phytic per 100 g flour	% decrease
0	4	0.890	-
1	2	0.205	77
2	2	0.205	77
3	2	0.205	77
4	2	0.205	77

N = number of experiments performed at the same time.

Table 5- Effect of carbon source on utilization of phytate by *S. cerevisiae*. The basic medium consisted of 5 g peptone & 3 g yeast extract per L (PYE).

Basic medium plus carbon source	mM PA	Growth in E at 550 nm	Found in medium	
			mM Pi	mM PA
8 % sucrose (120 h)	2.0	14.6±0.6	7.0±0.4	0.2±0.1
= (120 h)	3.0	10.8	0.6	2.7
= (120 h)	4.0	10.6	0.1	3.7
= (120 h)	5.0	11.2	0.4	4.8
8 % sucrose & 3 % ethanol	2.0	11.5	6.5	0.0
8 % sucrose ^a	5.0	10.9	13.0	0.3
8 % sucrose ^b	5.0	10.3	16.2	1.9
= (102 h)	10.0	13.9	18.5	3.9
= (102 h)	20.0	7.1	9.1	16.2
= (134 h)	40.0	5.5	12.1	35.1
3 % glucose ^c	2.0	15.7	0.3	1.8
3 % glycerol & 3 % ethanol ^c	2.0	0	NC	NC
3 % glucose & 3 % ethanol ^c	2.0	7.4	0.0	2.0

a- Culture medium was 200 ml in 3-L flask. Incubation period was 139 h; yeast inoculum was from culture adapted to 5 mM PA.

b- Inoculum from experiment-a in 30 ml medium; incubation period was 94 h.

c- Incubation period was 120 h. NC = no change.

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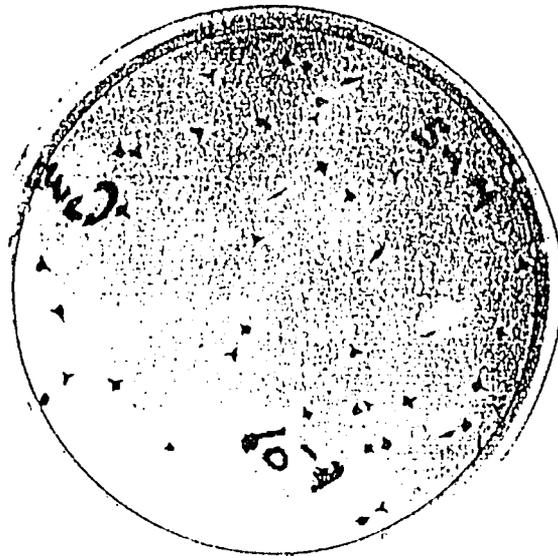


Plate 1- Y-5-a and Y-5-c colonies. *Saccharomyces cerevisiae* cells were first inoculated into Phytate-containing medium (5 mM IP6) at 28°C and the culture was agitated in a water shaker bath. The cells from this culture were then serially diluted and plated on agar plate containing 5 ml phytate. In the plate two colonies with tri-dentate (deltoid) (Y-5-a) and a crescent-like or concave (Y-5-c) morphology are seen.

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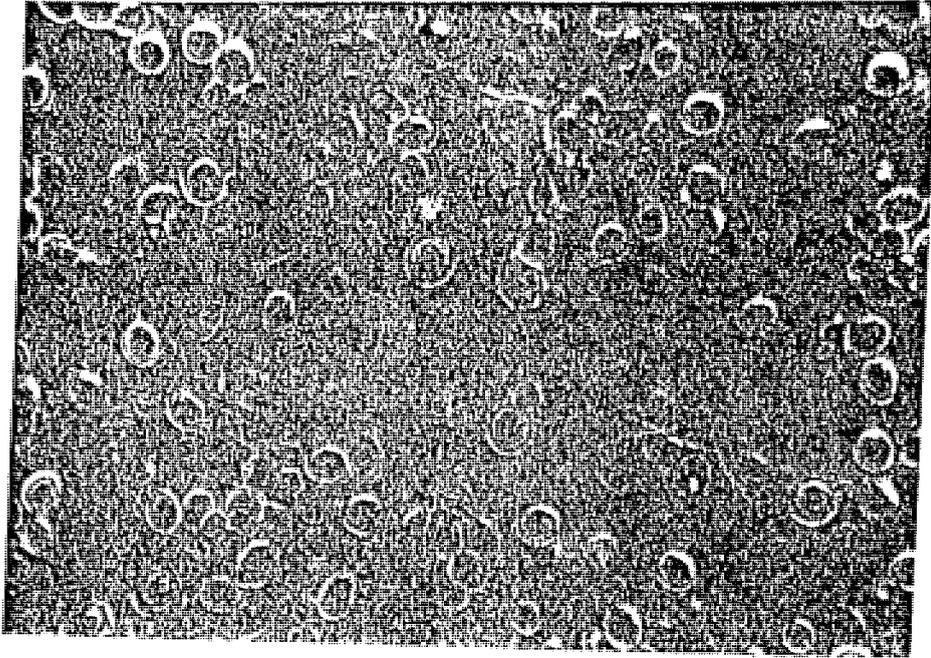
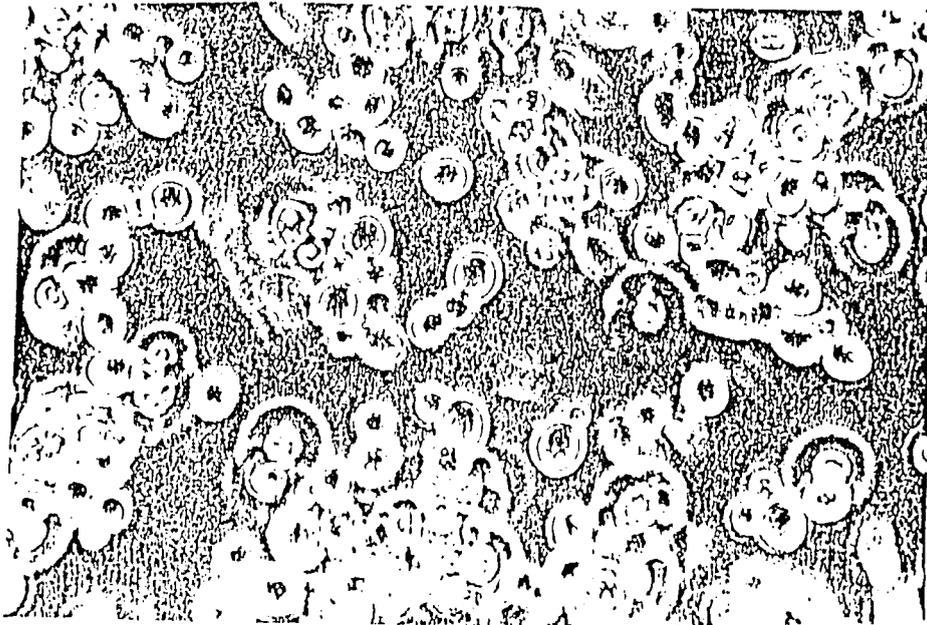


Plate 2- Live Y-5-a cells as visualized by methylene blue. These are typical cells which were grown on high levels of phytate (10 -40 mM) in YNB media. The plate shows that only yeast cells are present with numerous buds as means of cell division.



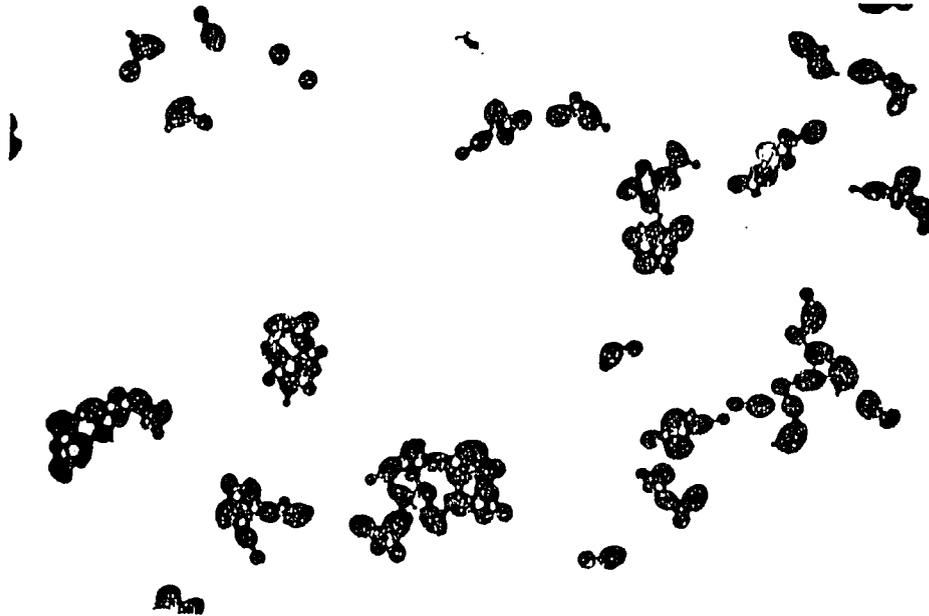


Plate 3- Y-5-a cells as visualized by Gram stain. These are typical cells which were grown on high levels of phytate (10 - 40 mM) in YNE media. The plate shows that only yeast cells are present with numerous buds as means of cell division.

6) Impact, Relevance and Technology Transfer:

The findings of this work are useful in the development of baker's yeast to be used in half-leavening dough in bread-making industry. To have this type of bread (or pastries) is of great importance in order to maintain the nutrition quality in the bread, the flavor and the availability of the essential elements: zinc, iron, and calcium. These cannot be achieved satisfactorily through the use of the currently applied commercial baker's yeast or through prolongation of fermentation period. We should not forget that the main source of food in Jordan as well as in the other developing countries is the whole wheat flour (and rice). Developed countries also use the products of this source of food. Therefore, the project is internationally useful. The results are encouraging since the strain we have isolated does degrade the phytate at low and high concentrations, that are found in the grains. The results in this report are not yet finalized, however. There are studies that have to be carried out 1) Application of the strain to the dough at laboratory scale level & then at large industrial scale by the bakeries, and examination of its effect on the degradation of the phytate within reasonable time of fermentation. 2) Studies on the stability of the strain in the process of fermentation of the dough, i.e. does it remain producing the enzyme(s) or not?. If the strain does not remain producing the enzyme(s), and this is expected because the other organophosphate substances in the wheat may be better phosphate source than phytate, genetic manipulation on this strain must be then carried out. Nevertheless and at this stage, the project has clarified the role of yeast in degradation of phytate in the dough, which was up to date nothing. The project has brought us closer to the solution of the problem. We now know that the phytate enters the cells, and in response to absence or very low inorganic phosphate, the strain produces the enzyme(s) that could degrade the phytate (i.e. enzyme(s) induction). This is the difference that the project has made. Although we are not fully equipped, we are now better equipped & qualified for carrying the research. We have gained the know-how techniques in dealing with yeast which is highly important microorganism in food technology. Research skills by the technicians, graduate students and senior investigators have greatly improved through this project. Also the project has enhanced the research and improved its quality through the equipment we had brought from the funds, namely, light microscope, autoclave & sterilizer, ashing oven, double beam spectrophotometer, refrigerated high speed microcentrifuge, cell disintegrator (polytron), deep freezer, water bath shaker and incubator, camera for instant photography for documentation, and other miscellaneous items that are essential for the research. We are still working on the project, and we are confident that we might have a breakthrough in this problem.

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7) Project Activities / Outputs:

No meetings were attended. A number of publications (at least two) are in preparation. Reprints will be submitted when results are published.

8) Project Productivity:

The proposed goals at this stage of the project have been accomplished. However, the ultimate goal of the project which is the production of bread that is of specific qualities as indicated in the original proposal has not been accomplished for a number of reasons. 1) Greater funds and longer period of time for conducting this type of research are needed. 2) The research has entered a stage that requires molecular biology and genetic manipulations. We are now pretty sure that the enzyme(s) that degrade phytate is/are inducible in the strain we have isolated. But there is a problem and that is enzyme induction would be suppressed up on the absence of the inductive conditions which are the phytate concentration and the medium composition. However, the probability that the strain would act on the phytate in the dough is there, & this is an experiment which has not been conducted yet for time limits on this phase of the project.

9) Future Work:

The project has already led us to a new phase of research in this problem. We are currently experimenting the new strain in dough leavening and its effect on the phytate level in the dough after fermentation. Also we are thinking of ways of genetic manipulation of the strain, and that is how to make the strain secrete the enzyme continuously under the conditions of dough-making. Of these ways is the use of and the introduction of a promoter into the gene of the enzyme of the strain. There are many publications concerning this type of research; and many products are made through this type of genetic engineering.

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