Phytate Degradation by Lactic Acid Bacteria and Yeasts during the Wholemeal Dough Fermentation: a ^{31}P NMR Study

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myo-Inositol hexaphosphate (IP6) is the main source of phosphorus in cereal grains, and therefore, in bakery products. Different microorganisms such as yeasts and lactic acid bacteria have phytase enzymes able to hydrolyze IP6 during the wholemeal breadmaking. In this paper, the phytase activity of *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus curvatus*, and *Saccharomyces cerevisiae* strains, isolated from southern Italian sourdoughs, is assayed using the ^{31}P NMR technique. The sourdough technology based on the use of lactic acid bacteria in the breadmaking is finally suggested.

KEYWORDS: Phytate degradation; ^{31}P NMR; bread; lactic acid bacteria; yeasts

INTRODUCTION

myo-Inositol hexaphosphate (IP6), largely known as the storage form of phosphorus in seeds, is particularly abundant in many cereal grains, oilseeds, and legumes and is contained in flours and brans of different cereals. IP6 is often reported as antinutrient (*1–3*) since it forms complexes with dietary minerals hindering their absorption and hence reducing their bioavailability (*4–7*). On the other hand, it is also reported that lower inositol phosphate derivatives can have health benefits in the protection against colon cancer (*8, 9*), arteriosclerosis, neural tissue, and coronary heart diseases (*10, 11*).

Phytase enzymes, widely present in organisms such as plants, microorganisms, and animal cells (*12*) make available minerals (*7, 13–15*) and phosphorus present in phytates through a stepwise IP6 hydrolysis.

The degradation of IP6 can be catalyzed either by endogenous enzymes, naturally present in cereals, or by microbial contaminant enzymes. The phytase activity, detected and studied in many microorganisms, depends largely on the biochemical characteristics of the enzymes (*16–20*).

Many bakery products such as bread have a significant content of IP6 (*21*), which can be hydrolyzed by microorganisms. Specifically, during the bread production, endogenous (from cereals) and microbial (yeasts or/and lactic acid bacteria naturally present in flour or added as starter) phytase enzymes are both active. In fact, during cereal processes such as malting, fermenting, and soaking, phytase enzymes catalyze the stepwise hydrolysis of IP6 to *myo*-inositol via penta- to monophosphates and orthophosphate (*2*).

Nowadays, the main baking technologies use both sourdough and baker's yeasts. In the modern baking industry, sourdough

processes are used to obtain bakery products with an excellent sensory quality and a prolonged shelf life, but they require a long fermentation time and a sophisticated technology. On the other hand, the baker's yeast technology obtains standard products in short fermentation times. It is important to investigate how these two different technologies can influence the metabolic activity of the microorganisms, and in particular, the phytase activity.

The phytase activity can be studied by measuring the amount of IP6 during fermentation. Different analytical methods such as ion-exchange chromatography (*22, 23*), complexometric titration (*21*), and ^{31}P NMR technique (*24, 25*) have been developed to determine the amount of phytates in food.

In this paper, the phytase action of five different microorganisms (i.e., yeasts and lactic acid bacteria isolated from Southern Italian sourdoughs) was studied using a suitable ^{31}P NMR technique. The phytase activities of lactic acid bacteria and yeasts were analyzed and compared.

MATERIALS AND METHODS

Breadmaking Procedure. Doughs were prepared by mixing whole-wheat flour (100 g), distilled water (approximately 60 mL), and starter cultures (10^6 cfu/g yeasts or 10^7 cfu/g lactic acid bacteria). Dough (A), used as control sample, was made without the starter culture addition. For the inoculum, strains from the DISTAAM collection of the University of Molise were used: all the strains were previously isolated from Southern Italian sourdoughs.

Five different strains were assayed: *Lactobacillus plantarum* (B), *Lactobacillus brevis* (C), *Lactobacillus curvatus* (D), and *Saccharomyces cerevisiae* strain 1 (E) and strain 2 (F). Cultures of lactic acid bacteria and yeasts were propagated routinely in MRS broth (*26*) and in YPD (10 g/L yeast extract, 20 g/L bacteriological peptone, 20 g/L glucose), respectively, for 24 h at 28 °C. Cells used for dough fermentation were incubated until the late exponential phase of growth was reached (~16 h). The fermentation of each dough was performed for 36 h at 28 °C.

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Phytate Degradation by Lactic Acid Bacteria and Yeasts

J. Agric. Food Chem., Vol. 52, No. 20, 2004 **6301**

Phytate Extraction. To study the kinetics of the phytate hydrolysis in the control sample and in doughs inoculated with microorganisms, the samples were withdrawn after 0, 2, 4, 6, 8, 12, 24, and 36 h of fermentation. At each sampling time, the pH of the dough was measured. The pH measurements were performed using a spin electrode pH meter (Crison model 2001). Phytate extractions were performed following the method of Kemme (*24*) with some modifications. Specifically, 6 g of dough sample was mixed with 30.0 mL of 0.75 M HCl by agitation for 2 h at 20 °C. The slurry was centrifuged at 1800*g* for 15 min, and 6.0 mL of the clear supernatant was heated for 15 min at 100 °C, under agitation. After cooling, the sample solution was centrifuged (1800*g* for 15 min). Subsequently, 8.0 mL of ethylenediaminetetracetic acid (EDTA: 30 mg/mL) aqueous solution was added to 4.0 mL of the supernatant. The pH of the solution was adjusted to 6.0 using 2 M NaOH; then, the sample was freeze-dried and dispersed in 5 mL of water and filtrated (0.45 μm). The pH was adjusted to 12.6 using NaOH. Finally, the samples were freeze-dried again.

NMR Analysis. Freeze-dried samples (100 mg) were dissolved in D_2O (600 μL) adjusting the pH to 12.6 since at this pH the inositol species are stable (*24*). ^{1}H and ^{31}P NMR spectra were recorded at 300 K on a Bruker AVANCE AQS600 spectrometer operating at 600.13 and 242.94 MHz, respectively. ^{1}H decoupled ^{31}P NMR spectra (*27*) were performed using a 5 mm broadband probehead with a 20 kHz spectral width, 8000 data points, a relaxation delay of 7 s, a GARP pulse sequence for proton decoupling, a number of scans of 512, and a 90° pulse on ^{31}P of 10 μs. An exponential multiplication (2 Hz line broadening) and zero filling to 16000 points were applied before the Fourier transformation. The ^{1}H decoupled ^{31}P inverse gated NMR spectrum[27,28] was obtained using a 45° pulse on ^{31}P and 30 s of relaxation delay. Chemical shifts for ^{31}P spectra are given in ppm with respect to an external standard of 85% H_3PO_4. The relative IP6 content is reported, in percents, as the ratio between the sum of the IP6 ^{31}P integrals and the sum of all ^{31}P integrals. The correct value of the Pi integral was obtained by introducing a correction factor, as described in the Results and Discussion.

The IP6 content in grams of P per kilogram of the freeze-dried extract was measured using 2-aminoethylphosphonic acid (9.4 mM) as an internal reference.

Because of the strong signal overlapping, the ^{1}H spectra of the extracts were not used to perform quantitative measurements.

The ^{1}H–^{31}P gradient-selected HMQC spectrum (*27–29*) was performed using a 5 mm Bruker multinuclear *z*-gradient inverse probehead. This experiment was performed in the echo–antiecho phase selective mode with the following parameters: a 90° ^{31}P hard pulse of 11 μs, 3 and 6 kHz spectral widths in proton and phosphorus dimensions respectively, 512 points in F_2, and 512 increments in F_1. Linear prediction up to 512 points was applied in F_1 dimension before Fourier transformation. Unshifted squared cosine window functions were applied in both dimensions.

T_1 relaxation time of ^{31}P nuclei was measured using a T_1 inversion–recovery experiment (*27*) with a relaxation delay of 60 s.

RAPD-PCR Analysis. The presence of the starter during the fermentation period was monitored by RAPD-PCR. RAPD-PCR was carried out both on strains used for inoculum and on strains isolated at the end of dough's fermentation. The identification of isolates was carried out on colonies chosen randomly.

Lactic acid bacteria cells were grown in 5 mL of MRS broth at 28 °C. Yeasts cells were grown in 5 mL of YPD medium (20 g/L bacteriological peptone; 20 g/L dextrose; 10 g/L yeast extract). Lactic acid bacteria DNA was extracted according to the method of Querol et al. (*30*), whereas yeasts DNA was extracted following the same method but using the lytic enzyme from *Rhizoctonia solani* (Sigma, St. Louis, MO) to digest the cell wall.

The amplification reactions were performed according to Andrighetto et al. (*31*) for lactic acid bacteria and to Succi et al. (*32*) for yeasts.

Lactic acid bacteria and yeast amplification products were separated by electrophoresis on 1.5 and 1.8% (w/v) agarose gel in 0.5 x TBE buffer, respectively.

Random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) profiles were obtained directly using the digital camera ImageMaster VDS (Amersham Pharmacia Biotech, Milan, Italy)

and analyzed with the pattern analysis software package Gel Compar Version 4.1 (Applied Maths, Kortrijk, Belgium). Similarity calculation in the profiles of bands was based on the Pearson product–moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method using the arithmetic average clustering algorithm (*33*).

RESULTS AND DISCUSSION

^{31}**P NMR Methodology.** Nuclear magnetic resonance is a powerful technique able to give us a relevant contribution in food analysis (*34*, *35*). A quantitative and extremely accurate ^{31}P NMR methodology for the determination of orthophosphate and inositol phosphates in diets was reported by Kemme et al. (*24*). The main advantages of the NMR technique in comparison to other techniques such as HPLC are that NMR allows one to detect and quantify all the phosphate compounds in the same experiment and that NMR does not need any standards. The NMR sources of bias influencing the recoveries of the total phosphorus content can be attributed to several factors involved in the sample preparation and to the low sensitivity of the NMR technique (*24*).

Here, further sources of error in the determination of phosphorus content are discussed. The quantification of the inositol phosphates and the ortophosphate can be obtained using the integral values of the ^{31}P resonances in the ^{1}H-decoupled ^{31}P NMR spectrum. To obtain correct integral values, the complete relaxation of ^{31}P resonances between consecutive scans has to be assured, and the NOE enhancement must be excluded; unfortunately, ^{31}P NMR experiments, performed using the inverse gated ^{1}H-decoupling and a long relaxation delay that fulfills completely both these conditions, require extremely long, often prohibitive, experimental times. Therefore, we decided to use the experimental conditions proposed by Kemme (*24*), namely, a GARP ^{1}H-decoupling and a relatively short relaxation delay of 7 s: in this way, more sensitive and less time-demanding experiments can be performed, but a correction factor has to be introduced to obtain a correct value of the phosphorus content.

The ^{31}P T_1 measurements showed that all ^{31}P resonances, except the orthophosphate resonance, have T_1 values short enough (1–2 s) to completely relax in our experimental conditions: on the contrary, the long T_1 value of orthophosphate resonance (about 7 s at pH 12.6) does not allow this resonance to relax completely. The incomplete relaxation of orthophosphate resonance between consecutive scans in ^{31}P NMR spectra results in an underestimation of its concentration. Nevertheless, the correct orthophosphate (Pi) concentration can be calculated by introducing a correction factor resulting from the ratio between the Pi resonance intensity in the inverse gated ^{1}H-decoupled ^{31}P spectrum of the control sample (with relaxation delay long enough for the complete orthophosphate relaxation) and the Pi resonance intensity in the GARP ^{1}H-decoupling experiment performed on the same sample under the typically used conditions (7 s relaxation delay). The intensity of IP6 resonances has to be set at the same value in these two spectra to compensate the NOE enhancement as well. In our experiments, the correction factor was equal to 1.5, and the Pi concentration was calculated by multiplying the integral value of the Pi resonance by the calculated correction factor. The error made in the estimation of the integrals was ~10% (*24*).

Finally, the correct quantification of Pi and IP6 in the extracts was obtained by using the relative concentrations of Pi and IP6 (see Materials and Methods) instead of their absolute concentrations. In fact, the results obtained using 2-aminoethylphosphonic
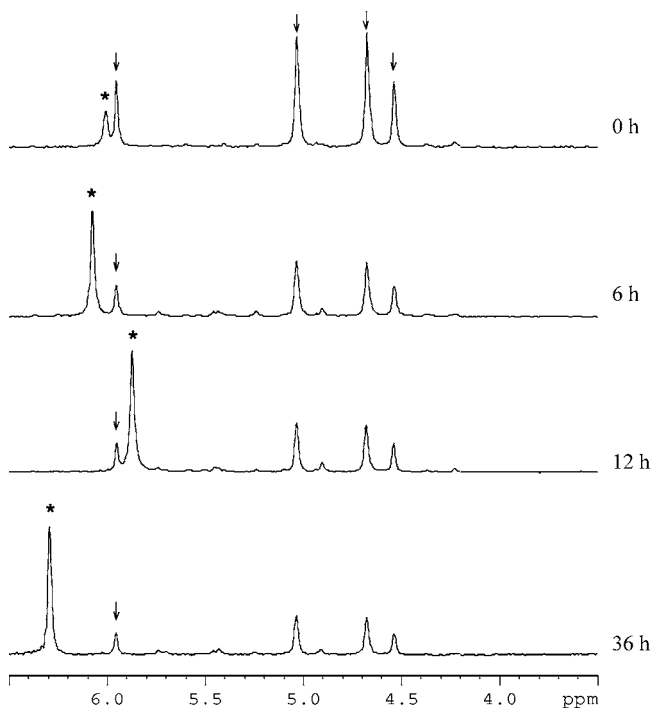
Figure 1. ^{1}H-decoupled ^{31}P NMR spectra of the extracts of wholemeal dough (control sample, **A**) at different sampling times, pH = 12.6 and T = 300 K. ↓ = IP6 resonances and * = Pi resonance.

Table 1. ^{1}H and ^{31}P NMR Assignments of IP6 in D_2O at pH 12.6 and $T = 300$ K^a

proton/phosphorus position in the IP6 moiety	^{1}H (ppm)	^{31}P (ppm)
1, 3	4.50	5.05
2	4.31	4.53
4, 6	4.59	4.70
5	4.50	5.95

^{a} ^{31}P chemical shifts are reported in ppm with respect to an external standard of 85% H_3PO_4. ^{1}H chemical shifts are reported in ppm with respect to a trace of DSS used as internal standard.

acid as an internal reference (*24*) in extracts of the same dough at different fermentation times showed that the overall quantity of phosphorus was not constant. The variation of total phosphorus content in these experiments was not systematical or monotonic; in fact, the total phosphorus content was in the range of 1.4−4 g of P per kilogram of the freeze-dried extract. The variation of absolute phosphorus content probably due to an incomplete extraction of inositol phosphates from wholemeal doughs (*24*) can give misleading results, especially when different extracts are compared.

Control Sample. ^{1}H-decoupled ^{31}P NMR spectra of the extracts of wholemeal dough (control sample) at different sampling times at pH = 12.6 in D_2O are reported in **Figure 1**. ^{1}H and ^{31}P resonance assignments, reported in **Table 1**, were performed using literature data (*24*) and a 2D HMQC experiment (data not shown). The 0.5 ppm downfield shift of all phosphorus resonances with respect to the literature data (*24*) is due to the different external reference. The ^{31}P assignment of IP6 resonances was also confirmed by the addition of sodium phytate to the control sample: the intensity of IP6 resonances increased, and no new lines were observed in the resulting spectrum.

The ^{1}H-decoupled ^{31}P spectrum at $t = 0$ of the control sample (**Figure 1**) shows clearly the IP6 resonances together with the Pi resonance. The ^{31}P spectrum of the sample obtained after 6
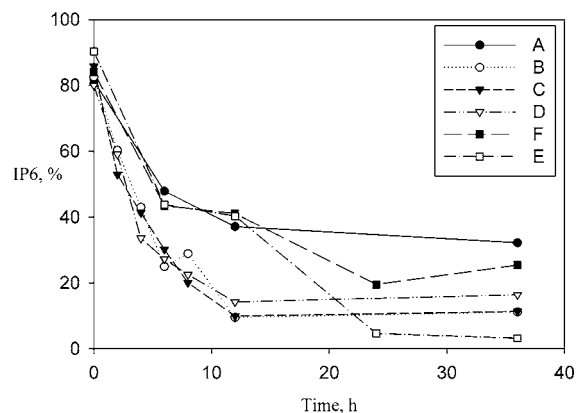

Figure 2. IP6 content (in % P total) in extracts of doughs after different fermentation times. The doughs were inoculated with *Lb. plantarum* (**B**), *Lb. brevis* (**C**), *Lb. curvatus* (**D**), *S. cerevisiae* strain 1 (**E**), and *S. cerevisiae* strain 2 (**F**). The IP6 content in the control sample (**A**) is also reported.

h of fermentation shows a clear increase of the Pi resonance and a lowering of the IP6 resonances. The kinetic curve shows that after 6 h about 40% of IP6 disappeared (see **Figure 2**). The degradation of IP6 can be the result both of the activity of endogenous phytase present in the flour and of the enzymatic activity of contaminant microorganisms. No new resonance due to intermediate compounds (i.e., IP5, IP4, IP3, IP2, and IP1) was significantly present: therefore, no accumulation of lower IPs was present in the control sample in agreement with the results obtained in the case of some feed ingredients (*24*). A possible explanation is that the phytases present in doughs are able to breakdown the intermediate IPs. Finally, in the case of the control sample, the enzymatic hydrolysis was not complete; in fact, after 36 h, a significant amount of IP6 was still present. It is possible that the released phosphate inhibited a further enzymatic activity.

Samples Inoculated with Lactic Acid Bacteria. The ^{1}H-decoupled ^{31}P NMR spectra of extracts of dough inoculated with *Lb. plantarum* are shown in **Figure 3**. During the fermentation, the IP6 resonances decreased, the Pi resonance increased, and the presence of some new resonances was observed. On the basis of the nonsimultaneous appearing of these new peaks during the fermentation, at least three different groups of peaks corresponding to three different lower inositol phosphates were distinguished. The comparison between these spectra and the literature data (*24*) suggested that the IP5 and IP4 were not present in our samples. Therefore, no accumulation of IP5 and IP4 was present, and the unknown resonances can be tentatively assigned to IP3−IP1 species. The incomplete hydrolysis of IP3−IP1 inositol phosphates suggested that the phytase activity of lactic acid bacteria exhibited a different substrate specificity with respect to that observed in the control sample.

The kinetic curves relative to the doughs inoculated with *Lb. plantarum*, *Lb. brevis*, and *Lb. curvatus* are reported in **Figure 2**. These three strains showed a phytase activity with a similar kinetic pattern; therefore, the enzymatic action of these lactic acid bacteria probably follows the same mechanism. The IP6 hydrolysis occurred in the first hours when a relevant decrement of IP6 intensity was observed; after 12 h, IP6 content was almost constant, suggesting an interruption of the hydrolysis.

Finally, the kinetic curves showed clearly that these bacteria induced a higher IP6 reduction, and consequently, a major phytase hydrolysis in comparison to that observed in the control sample (**Figure 2**); in fact, after 12 h, the kinetic curves showed that in doughs inoculated with lactic acid bacteria, about
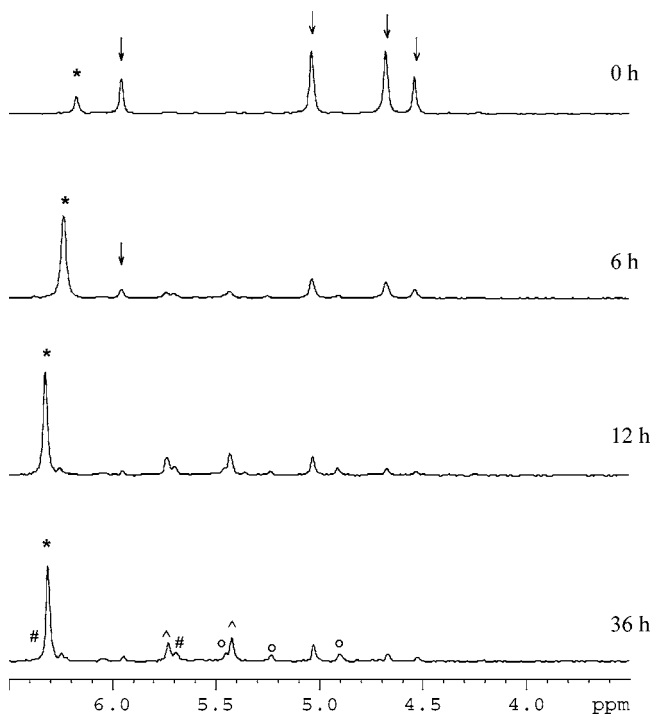
Phytate Degradation by Lactic Acid Bacteria and Yeasts

J. Agric. Food Chem., Vol. 52, No. 20, 2004 **6303**

Figure 3. ^{1}H-decoupled ^{31}P NMR spectra of extracts of dough inoculated with *Lb. plantarum* (**B**) at different sampling times, pH = 12.6 and *T* = 300 K. ↓ = IP6 resonances and * = Pi resonance. Symbols °, ∧, and # indicate the resonances of three different low inositol phosphates.
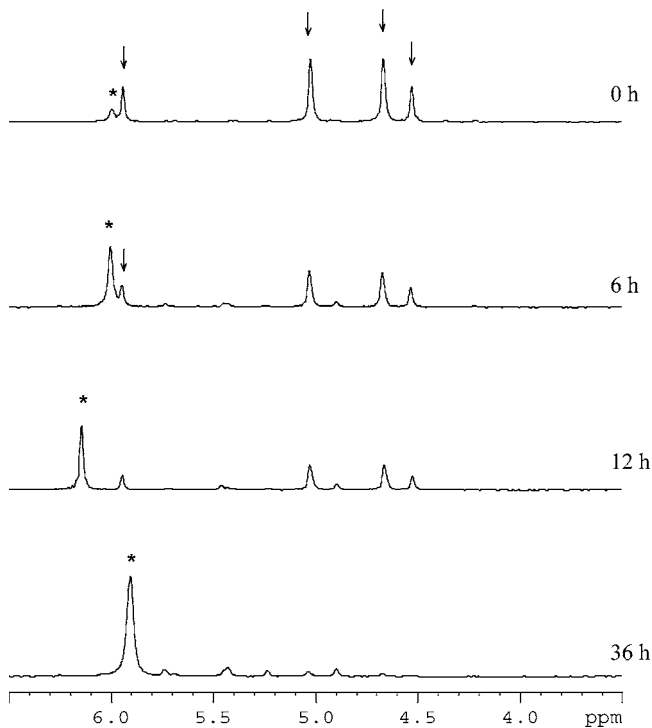

Figure 4. ^{1}H-decoupled ^{31}P NMR spectra of extracts of dough inoculated with *S. cerevisiae* strain 1 (**E**) at different sampling times, pH = 12.6 and *T* = 300 K. ↓ = IP6 resonances and * = Pi resonance.

80−90% of IP$_6$ disappeared, whereas in the control sample after the same time only 50% of IP6 was hydrolyzed.

Samples Inoculated with Yeasts. ^{1}H-decoupled ^{31}P NMR spectra of dough extracts inoculated with yeast E are shown in **Figure 4**. During the fermentation, a clear decrease of the IP6 resonances together with an increase of the Pi resonance were

Table 2. Phytase Activities of Lactic Acid Bacteria and Yeasts after 36 h of Fermentation[a]

inoculated sample[b]	IP6 breakdown (%)
B	21
C	21
D	16
E	29
F	7

[a] Intrinsic phytase activity of the wholemeal dough contribution is excluded. [b] B = *Lb. plantarum*; C = *Lb. brevis*; D = *Lb. curvatus*, E = *S. cerevisiae* strain 1; and F = *S. cerevisiae* strain 2.

observed; no significant accumulation of lower IPs intermediates was observed.

The kinetic curves relative to the IP6 hydrolysis in doughs inoculated with yeasts E and F are reported in **Figure 2**. It is important to notice that in the case of yeasts, after 12 h only 50% of IP6 degradation occurred, whereas in the case of lactic acid bacteria after the same time, 80−90% of IP6 disappeared, suggesting that the degradation rate of IP6 in the case of yeasts was slower than that observed in the case of lactic acid bacteria. The high degradation rate in the case of lactic acid bacteria is probably due to the suitable pH condition for the IP6 degradation reached in the first hours of the fermentation (see the following section).

Moreover, the IP6 amount was constant after 24 h. In the case of yeast E, the total IP6 degradation was reached: in fact, at the end of the fermentation, the IP6 intensity was almost equal to zero. On the other hand, in the case of yeast F, IP6 did not disappear completely, and about 25% of IP6 respect to the initial amount was still present at the end of the fermentation.

Finally, the phytase activities of yeasts and acid bacteria as obtained by the subtraction of the background IP6 breakdown of each inoculated sample from IP6 breakdown of the control samples after 36 h of the fermentation are reported in **Table 2**. In this way, the phytasic activity of yeasts and lactic acid bacteria was evaluated without the contribution of the intrisic phytase activity, which was the same in all the samples: the lactic acid bacteria B, C, and D had a similar phytase activity, and yeast F showed a very low phytase activity, whereas yeast E had the highest IP6 breakdown.

pH Measurements. The phytate degradation rate depends on the pH conditions (*1, 19*); suitable pH values for the IP6 degradation are in the 4.5−5.5 range (*1*). The pH values of doughs during the fermentation are reported in **Figure 5**. As expected, during the fermentation, doughs inoculated with lactic acid bacteria (B, C, and D) showed lower values of pH in comparison both to the control sample (A) or to the doughs inoculated with yeasts (E and F). The lowering of the pH from 6 to 5 in the case of lactic acid bacteria could be responsible for the high degradation rate reached in the first hours of the fermentation. On the other hand, in the case of samples E and F, the pH values were constant in the first hours of the fermentation, and only at the end of the fermentation were pH values around 5 reached: this is in agreement with the observed slow degradation rates observed in the case of samples inoculated with yeasts.

RAPD-PCR. To confirm the presence of the starter at the end of the fermentation, the microbiological composition of the inoculum and of the mature doughs (36 h) was compared. Each starter strain and some strains randomly isolated from the inoculated samples were screened by DNA analysis (RAPD-PCR). The genotypic identification showed high similarities between the strains used for inoculum and those isolated from
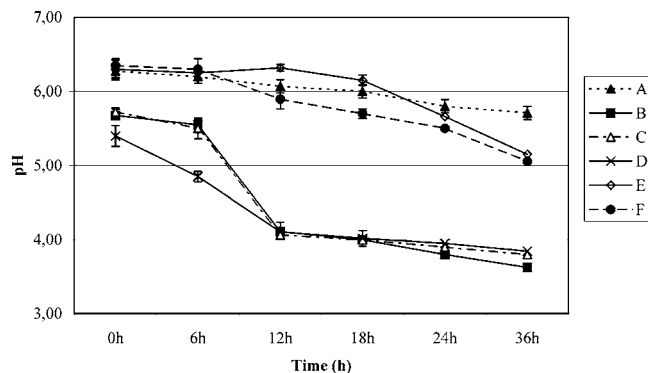
6304 *J. Agric. Food Chem.,* Vol. 52, No. 20, 2004

Reale et al.

Figure 5. pH values of doughs during the fermentation: control sample (**A**), *Lb. plantarum* (**B**), *Lb. brevis* (**C**), *Lb. curvatus* (**D**), *S. cerevisiae* strain 1 (**E**), and *S. cerevisiae* strain 2 (**F**). Each point represents the mean of three measurements made in different parts of the sample. Vertical bars indicate the standard deviation.
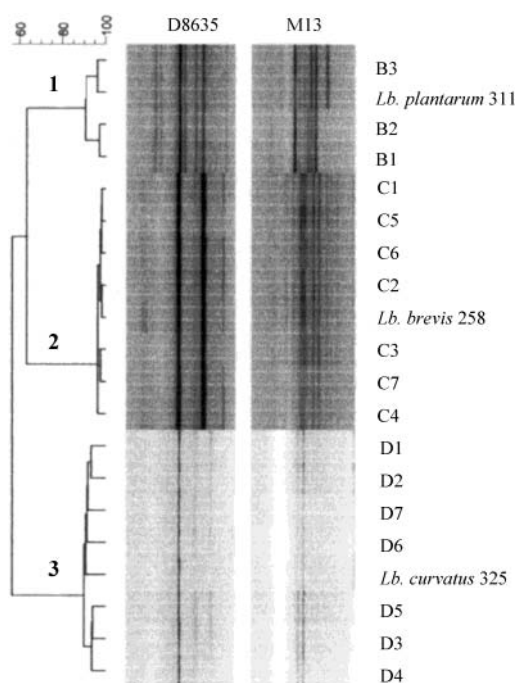

Figure 6. RAPD profiles of starters used for inoculum and lactic acid bacteria isolated at the end of the fermentation of doughs. Cluster 1: *Lb. plantarum* starter strain (311) and *Lb. plantarum* isolated strains (B1–B3); cluster 2: *Lb. brevis* starter strain (258) and *Lb. brevis* isolated strains (C1–C7); and cluster 3: *Lb. curvatus* starter strain (325) and *Lb. curvatus* isolated strains (D1-D7).

samples at the end of fermentation. **Figure 6** shows the RAPD-PCR profiles both of the lactic acid bacteria used as starters and of the bacteria isolated at the end of the fermentation. The resulting dendrogram shows clearly the presence of three clusters: cluster 1 groups *Lb. plantarum* starter strain (311) and three strains (B1, B2, B3) randomly isolated at the end of fermentation of dough B; cluster 2 groups *Lb. brevis* starter strain (258) and seven strains (C1−C7) randomly isolated at the end of fermentation of dough C; cluster 3 groups *Lb. curvatus* starter strain (325) and seven strains (D1-D7) randomly isolated at the end of fermentation of dough D. The profiles of the bands in each cluster show no differences between the starter and the corresponding strains isolated at the end of fermentation. This result confirms the presence of the starters at the end of the fermentation. The same results were obtained in the case of the yeasts (data not shown).

In the case of the main bakery products such as bread made with baker's yeast, the time required for the fermentation time is ~2 h: this means that if the activity of all yeasts used in breadmaking was similar to that observed in the case of yeasts E and F, the IP6 present in flours cannot be significantly degraded during the breadmaking. On the other hand, bakery products made with sourdoughs require a long fermentation time, thus allowing a significant IP6 degradation. So, taking into account the fermentation times used in breadmaking tecnologies and the specific phytase activity of the starters used for breadmaking, the sourdough technology based on the use of lactic acid bacteria seems to be more suitable for producing bread and other bakery products with an improved minerals bioavailability and a higher content of lower IPs with health benefit properties. Further studies involving the action of other microorganisms are in progress.

ABBREVIATIONS USED

IP6, *myo*-inositol hexaphosphate; IP5, *myo*-inositol penta-phosphate; IP4, *myo*-inositol tetraphosphate; IP3, *myo*-inositol triphosphate; IP2, *myo*-inositol diphosphate; IP1, *myo*-inositol monophosphate; Pi, orthophosphate.

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