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Safety evaluation of xylanase preparations: Acute and subchronic oral studies in Wistar rats

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ABSTRACT

A study was conducted to establish the toxicity evaluation of xylanases from *Penicillium occitanis* Pol6 and the expressed ones in the methylotrophic yeast *Pichia pastoris* to be used as a processing aid in the baking industry. The dose delivered in the acute study was therefore 2000 mg/kg body weight samples powder. The subchronic study of these xylanases was performed in Wistar rats at a dose level of 12000 UTX/kg body weight/day (this dose is three times the maximum dose of xylanase to be expected in bakery products). Body weight, food and fluid intake, antioxidant enzyme activities, biochemical parameters in plasma and urine and hematological parameters were obtained. Histopathology by light microscopy was performed. Furthermore, the expressed xylanase preparation was found not to represent mutagenic or clastogenic potential in the bacterial reverse mutation assay. According to the results of this study, the studied xylanases possess no discernible risk to human health and no adverse effects were detected by oral administration.

KEYWORDS

Xylanase;
Penicillium occitanis Pol6;
Pichia pastoris;
Safety evaluation.

INTRODUCTION

Xylanases are xylan-hydrolysing enzymes that occur naturally and are widespread in plants and microorganisms. The systematic name of the xylanase that is the subject of this article is 1,4-β-D-Xylan xylanohydrolase. It has the CAS No. 9025-57-4 (xylanase, endo-1,4) and the EC classification EC 3.2.1.8. In addition to these classifications, xylanases are further classified as glycosyl hydrolases family 10 (F) and family 11 (G) xylanases[9]. The family 10 xylanases differ from the family 11 xylanases primarily with regard to size, and the family 10 xylanases have relatively higher molecular weight than the family 11 xylanases. Xylanases used in food production generally belong to family 11, to which the subjects of this

article also belong.

The *Penicillium occitanis* Pol6 mutant secreted two xylanases termed PoXyn3 and PoXyn2; these two enzymes were successfully expressed in the methylotrophic yeast *Pichia pastoris* X-33 under the control of the glyceraldehyde 3-phosphate dehydrogenase (GAP) constitutive promoter. These two enzymes were purified to homogeneity by a simple, one-step purification protocol. The PoXyn2 mature protein is 320 amino acids long and has a calculated molecular weight of 29.88 kDa[12], while PoXyn3, with the lower molecular weight, mature protein presents 197 amino acids long and 21.73 kDa, in good agreement with the values determined for the native PoXyn3[11]. Even though the two xylanases genes products are completely characterized in the transgenic products, this does not necessarily provide information on their potential toxicity[21,28].

The xylanases discussed herein are intended for use as a processing aid in the milling and baking industry. In these industries, xylanases are used as dough strengtheners since they provide excellent tolerance to the dough towards variations in processing parameters and in flour quality. They also significantly increase volume of the baked bread[12]. Thus, for, care should be taken for consumer application of microbial enzymes in food processing safety. European Union (EU) recommends selecting carefully the nature and extent of toxicological studies performed in novel foods or ingredients, taking into account the source and composition of the novel food, its potential intake, and whether it is intended for a specific application or for more general use in the diet[13]. Data obtained from short-term studies are more useful because they help to determine the potential adverse effects of the compound at low enough doses to allow survival of the animals after a longer exposure period.

The overall objective of this investigation was first to study the effects of two xylanases from *P. occitanis* and the expressed ones in *P. pastoris* administrated by oral route to adult rats and second to identify the lowest-observed-adverse-effect levels (LOAELs) and the no-observed-adverse-effect levels (NOAELs) for acute and subchronic xylanases exposure.

MATERIALS AND METHODS

Chemicals

Glutathione (oxidized and reduced), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5-52 -dithio-bis-2-nitrobenzoic (DTNB) and thiobarbituric acids (TBA) were purchased from Sigma (St. Louis; MO, USA). All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

Strains and culture conditions

The *Penicillium occitanis* Pol6 mutant was supplied by Cayla Co. (Toulouse, France). The Pol6 strain is an hypercellulolytic mutant that was selected after eight rounds of mutagenesis from the CL100 wild-type strain[18]. The fungus was cultivated at 30 °C for seven days in a liquid medium containing Oat spelt xylan as a carbon source. Erlenmeyer flasks were used for 1 L of solution containing glucose (2% w/v) and 200 ml of Mandel's medium[23] (in g/l): KH_2PO_4, 2; $NaNO_3$, 5; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; yeast extract, 1 g; trace elements solution, 1 ml/l; and Tween 80, 1 ml/l. This solution was inoculated with a mycelium suspension from a routine subculture. The solution of trace elements contained (in g/l): $CoCl_2$, 2; $MnSO_4 \cdot H_2O$, 1.6; $ZnSO_4 \cdot H_2O$, 1.4; and $FeSO_4 \cdot 7H_2O$, 5.0. The pH of the culture was adjusted to 5.5 with NaOH (10 N)[7].

The *P. pastoris* host strain was X-33 (wild-type strain from Invitrogen, San Diego, CA). The recombinant *Pichia pastoris*, with either PoXyn2 or PoXyn3, were grown in buffered YPD medium containing 10 g yeast extract, 20 g Bacto-peptone, 20 g D-glucose, and 10 mmol sodium phosphate buffer with the pH initially adjusted to 6.8.

Preparation and characterization of the test articles

The test substances used for the toxicological investigations described in this paper are liquid enzyme preparations. The enzyme activity is expressed by its ability to degrade xylan. The xylanase enzyme hydrolyses the substrate to form reducing sugars which are quantified by the DNS method[2]. The β-xylanase unit is named TXU (Total Xylanase Units).

The *P. pastoris* strains X33; a commercially available protease-deficient was transformed with genes of xylanase 2 and xylanase 3 from *P. occitanis* Pol6. Individual transformants, grown on YPD medium, were screened for maximum expression of active recombinant xylanases. This screen resulted in the selection of the production strains. These two recombinant strains,

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combined together, consist the first test article designated below as xylanases of group IV. On the other hand, the native xylanases (PoXyn2 and PoXyn3) produced on Mandels and Weber medium by *P. occitanis* Pol6 was also prepared as the second test article and designated as xylanases of the group II.

The two test articles were produced from original strains from a frozen glycerol stock by fermentation in accordance with current good manufacturing practices (cGMP) for foods.

The fermentation broths were centrifuged to remove most of the initial cell mass (>99%). The process stream was then clarified using a plate and frame filter press to remove any remaining cells. The test article materials were lyophilized to increase shelf life. The materials were stored at 4–8 °C in sterile PETG bottles sealed under nitrogen. The final test batches appear as clear liquids at room temperature, completely miscible with water and do not contain stabilizers or preservatives.

Prior to use in toxicological studies, the two test articles were analyzed for chemical and microbial composition, including the absence of the producing strains. The results demonstrated that the samples conformed to the microbial and chemical specifications established for enzyme preparations used in food processing, as published in the Food Chemical Codex (FCC). Key characteristics of the test materials are given in TABLE 1.

TABLE 1 : Characteristics of the xylanases from the two test articles.

	Test articles	
	Group II	**Group IV**
Enzyme activity[a] (TXU/ml)	19879.45	6789.43
Dry matter content (%)	18.54	11.45
Carbohydrate (anthron) g/kg	12.4	45.6
Water (% w/w)	83.5	87.6
Total organic solids (% w/w)	13.4	12.3
N_{tot} (% w/w)	1.47	1.36
Ash (% w/w)	<0.1	<0.1
Total viable count	-	10^9-10^{11}
Penicillium occitanis Pol6 (production strain detection)	Not detected	-
Pichia pastoris (production strain detection)	-	Not detected
Antimicrobial activity	Not detected	Not detected

[a] **The activity of the xylanase is defined in TXU (total xylanase units). The xylanase activity was determined at 45°C in 50 mM sodium-acetate buffer, pH 5.5, using the modified method of Bailey et al, 1992. The specific activity of the xylanase 2 and 3 are 15,000 and 23,000 TXU/ mg, respectively using this assay.**

Animals and treatment

Male rats of Wistar strain with initial mean body weight of 130 ±10 g, obtained from the Central Pharmacy (SIPHAT. Tunis. Tunisia), were used in this study. They were housed at ambient temperature 22±3°C in a 12-hour light/dark cycle and a minimum relative humidity of 40%. The animals had free access to commercial pellet diet (SICO, Sfax, Tunisia) and water *ad libitum*. The composition of basal diet was described in TABLE 2. The general guidelines for the use and care of living animals in scientific investigations were followed[8]. The handling of the animals was approved by the Tunisian Ethical Committee for the Care and Use of laboratory animals.

One week after acclimatization, thirty five rats were randomly divided into five groups of seven animals each:

group I served as controls which received standard diet and pure drinking water;

group II received via drinking water native xylanases juice from *P.occitanis* Pol6 at dosage of 44.87 ml/ kg body weight /day

TABLE 2 : Composition of standard rat diet. Total energy (kJ/kg) 16297

Ingredients [a]	Amount in control diet(g/kg)
Casein	200
DL-Methionine	3
Corn starch	393
Corn oil	155
Sucrose	154
Cellulose	50
Mineral mix [b]	35
Vitamin mix [c]	10

[a]**All ingredients were purchased from the Industrial Society of Nutrients (SICO, Sfax,Tunisia). The diet is designed for laboratory animals (primarily for rats and mice) raised in laboratory conditions and used for experiments. All the remaining data are given according to the producer;** [b]**Mineral mixture contained the following (mg kg-1 of diet): $CaHPO_4$, 17200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_2, 120; $FeSO_4 \cdot 7H_2O$, 200; trace elements, 400 ($MnSO_4 \cdot H_2O$, 98; $CuSO_4 \cdot 5H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 80; Na_2SeO_4, 0.17; $CoSO_4 \cdot 7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g [per kg of diet]);** [c] **Vitamin mixture contained the following (mg kg-1 of diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin (vitamin B_{12}), 0.1; vitamin E (α-tocopherol acetate), 100; menadione, 80; nicotinic acid, 200; choline, 2720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0.6.**

which is equivalent to12000 UTX/kg body weight/day;

group III received orally the Mandels and Weber medium not been inoculated with *P.occitanis* Pol6;

group IV received via drinking water expressed xylanase (PoXyn2 and PoXyn3) juices from recombinant *Pichia pastoris* at dosage of 87.56 ml/ kg body weight /day which is equivalent to 12000 UTX/kg body weight/day;

group V received orally the YPD medium not been inoculated with *P.pastoris*.

The present study was designed to investigate the toxicity of xylanases PoXyn2 and PoXyn3 administrated to rats during 45 days. The xylanase doses and the treatment period were selected on the basis of previous studies[19].

During the experimental period, food and water intake were monitored daily. The amount of ingested diet was calculated as the difference between the weight of feed that remained in the food bin (Da) and the amount placed one day before (Db). These data were then used to calculate the daily average feed intake, according to the formula:

Average feed intake = (Db — Da)

Quantities of xylanases ingested by each rat were calculated from water intake, respectively (TABLE 3).

Urinary samples, collected into bottles within 24-h cycles, were obtained from each animal housed in a specially designed metabolic cage where faecal contamination was avoided.

At the end of the experimental period, animals of different groups were killed by cervical decapitation to avoid stress. Blood was collected in heparinised tubes and centrifuged at 2200g for 10 min. Plasma samples were drawn and stored at -80 °C until analysis. Other blood samples were collected in 5 ml test tubes containing EDTA for the determination of the hematological parameters within the next twenty four hours. Selected organs for weight were dissected out, cleaned and weighed. Some kidney samples, immediately removed, were cleaned and fixed in 10% formalin solution for histological studies.

Acute toxicity test

Animals were fasted for 16 h prior to dosing. Male rats were dosed by oral gavage, using a stainless steel feeding needle, with 20 ml/kg body weight of aqueous solution containing 10% (w/v) of both xylanase powder from both *P. pastoris* and *P. occitanis* strains. The dose delivered was therefore 2000 mg/kg body weight samples powder. All rats were monitored continuously for 24 h after dosing for signs of toxicosis.

TABLE 3 : Daily food intake and water consumption.

Groups	Food Consumption (g/rat/day)	Water Consumption (ml/rat/day)	Ingested quantities of Xylanases (TXU/ ay)
Group I	14.84 ± 1.15	30.59 ± 4.32	-
Groupe II	14.90 ± 1.38	32.39 ± 5.70	3237.74± 549.69
GroupeIII	15.02 ± 1.40	34.08 ± 5.79	-
Groupe IV	14.96 ± 1.23	31.75 ± 5.50	4105.55 ± 795.46
Groupe V	14.58 ± 1.25	30.71 ± 5.95	-

Values are means+SD for seven rats in each group.

Animals were monitored daily for any additional behavioral or clinical signs of toxicity and weekly for changes in body weight. Rats were euthanized on day 14 by lethal dose of sodium pentobarbital, and organs and tissues were examined macroscopically for toxicantinduced changes.

Biochemical assays

Protein quantification

Plasma protein content was determined according to Lowry et al.[22] using bovine serum albumin as a standard.

Determination of hematological parameters

White blood cells (WBCs), red blood cells (RBCs), hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were analyzed by an electronic automate Coulter MAXM (Beckman Coulter, Inc, Fullerton, California, USA).

Plasma lipid peroxidation

The concentration of malondialdehyde (MDA) in plasma, an index of lipid peroxidation, was determined spectrophotometrically according to Drapar and Hadley[10]. An aliquot of 0.5 ml of plasma was mixed with 1 ml of trichloroacetic acid solution and centrifuged at 2500g for 10 min. One milliliter of 0.67% thiobarbituric acid reagent (TBA) and 0.5 ml of super-

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natant were incubated for 15 min at 90 °C. The mixture was then cooled and the absorbance was determined at 532 nm using a spectrophotometer. Lipid peroxidation was expressed as ηmol of thiobarbituric acid reactive substances (TBARS), using 1,1,3,3-tetra-ethoxypropane as standard. Results were expressed as ηmol/mg protein.

Determination of protein carbonyls content

Protein carbonyls (PCO) were measured using the method of Reznick and Packer[30]. Briefly, 100 µl of plasma were placed in glass tubes. Then 500 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl were added. Tubes were incubated for 1 h at room temperature. Samples were vortexed every 15 min. Then 500 µl TCA (20%) were added and the tubes were left on ice for 5 min followed by centrifugation for 10 min. The protein precipitates were collected. The pellet was then washed twice with ethanol–ethyl acetate (v/v). The final precipitate was dissolved in 600 µl 6 M guanidine hydrochloride solution and incubated for 15 min at 37ºC. The absorbance of the sample was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2 \times 10^4$ cm^{-1} M^{-1}) and expressed as ηmoles/mg protein.

Determination of antioxidant enzyme activities

Catalase (CAT) activity was assayed by the method of Aebi[1]. Enzymatic reaction was initiated by adding an aliquot of 20 µl of the plasma and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer (pH 7.4). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of µmole H_2O_2 consumed/min/mg of protein.

Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich[3]. The reaction mixture contained 50 mM of plama in potassium phosphate buffer (PH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 µM ribofiavin and 75 mM nitro blue tetrazolium (NBT). The developed blue color in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units/mg of protein.

Plasma glutathione (GSH) levels

GSH in the plasma was determined by the method of Ellman[14] modified by Jollow et al. (1974). The method was based on the development of a yellow color when DTNB (5,5-dithiobtis-2 nitro benzoic acid) was added to compounds containing sulfhydryl groups. 500 µl of plasma were added to 3 ml of 4% sulfosalicylic acid. The mixture was centrifuged at 1600g for 15 min. Five hundred microliters of supernatants were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min. Total GSH content was expressed as µg/mg of protein.

Plasma ascorbic acid levels

Ascorbic acid determination was performed as described by Jacques-Silva et al.[20]. Protein in the plasma was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of sample (300 µl) was adjusted with H_2O to a final volume of 1 ml and incubated at 38 °C for 3 h, then 1 ml of H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and CuSO4 (0.075 mg/ml). The data were expressed as µmol of ascorbic acid/mg protein.

Biochemical parameters in plasma and urine

Albumine levels were determined in plasma of adult rats using kits from Biomaghreb (ref 20094). Creatinine and urea levels in plasma and urine were estimated spectrophotometrically using commercial diagnostic kits respectively (ref 20091, 20151) purchased from Biomaghreb (Ariana, Tunisia).

Histopathological studies

Kidneys, intended for histological examination by light microscopy, were immediately fixed in formalin solution (10%) and processed in a series of graded ethanol solutions. Then they were embedded in paraffin, serially sectioned at 5 µm and stained with hematoxylin–eosin. Three slides were prepared from each group.

Bacterial reverse mutation assay

A bacterial reverse mutation assay was carried out at Novo-zymes (Bagsvaerd, Denmark) in order to determine the ability of expressed xylanases to induce gene mutations in vitro[27]. Four histidine-requiring strains of *Salmonella typhimurium* (*S. typhimurium*) and one tryptophan-requiring strain of *E. coli* capable of detecting both induced frame-shift (TA1537 and TA98) and base pair substitution mutations (TA1535, TA100

and *E. coli* WP2 uvrA pKM101) were applied in this study. The genotypes of the bacterial test strains were conûrmed as described by Maron and Ames an d Green[17,24]. The study was carried out with and without the metabolic activation system S9 (MP Biomedicals LCC, Solon, Ohio) a liver preparation from male rats pre-treated with aroclor 1254 and the co-factors required for mixed function oxidase activity (S9 mix).

In a preliminary investigation it was demonstrated that the expressed xylanases preparation contained sufûcient amounts of histidine and tryptophan to signiûcantly support growth of the test strains using selective agar media. In order to avoid the risk of artefacts due to growth stimulation, a 'treat and plate' assay was applied[17] as described by Green[29]. The growth stimulation of the tryptophan-requiring *E. coli* strain is only weak and insigniûcant. Therefore, this part of the study was conducted by direct plate incorporation[24]. The test strains were exposed to the enzyme in liquid culture for 3 h and subsequently removed by centrifugation prior to plating on minimal glucose agar plates.

Two independent and identical experiments were performed. All bacterial strains were exposed to serial dilutions of expressed xylanases, solvent (sterile deionised water), and appropriate positive controls (see TABLE 9). The final concentrations of expressed xylanases were 2.5, 1.25 and 0.156 mg expressed xylanases dry matter per mL (*S. typhimurium*) or per plate (*E. coli*). For each 'treat and plate' assay with Salmonella strains, incubation mixtures were prepared in a series of sterile tubes. These consisted of 4 mL Oxoid nutrient broth No. 2, 1 mL bacterial culture, 1 mL test or control solution and 4 mL of either 0.2 M phosphate buffer (pH 7.4) or S9 mix. These mixtures were incubated for a period of 3 h at 37 °C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions twice in phosphate buffer and then finally resuspended in 2 mL of this buffer. The number of revertants per plate was determined by triplicate plating at each dose on selective agar as described by Maron and Ames[24] for the direct plate incorporation assay. Further, the number of viable bacteria in each culture was determined by plate count. The mean number of revertants per plate at each dose of the test substance was calculated and compared with the appropriate solvent control.

Statistical analysis

The data were analyzed using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). Statistical analysis was performed using one-way Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test as a post hoc test for comparison between groups. All values were expressed as means±S.D. Differences were considered significant if $p < 0.05$.

RESULTS

Acute toxicity test

We did not observe any signs of toxicity during the acute investigation. Normal tendency in body weight was observed and none of the tissues examined showed any significant changes.

Effects of xylanases on rats general health

Death was not observed in any experimental group during the treatment period (45 days). During the experiment, rats in the control group and treated group did not show any sign of toxicity.

Food intake

In treated rats, food intake and water consumption were not deviated when compared to controls (TABLE 3). Ingested xylanases quantities were similar in the different treated group. The high standard deviations on fluid consumption could be caused by the measuring method used

Evaluation of body, absolute and relative kidney weights

Mean body weight gain (Figure 1) and relative weights of some soft organs like liver, kidney, heart and spleen of the five groups were listed in TABLE 4. There were no significant differences ($p < 0.05$) in body weight gain and relative weights of these organs between controls and treated groups.

Hematological parameters

Compared with the control group, red blood cells' number (GR), hemoglobin concentration (Hb) and hematocrit (Ht) were unaffected in the treated groups. Other erythrocyte parameters such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), were also unchanged in treated rats. White blood cells still also unchanged compared with those of

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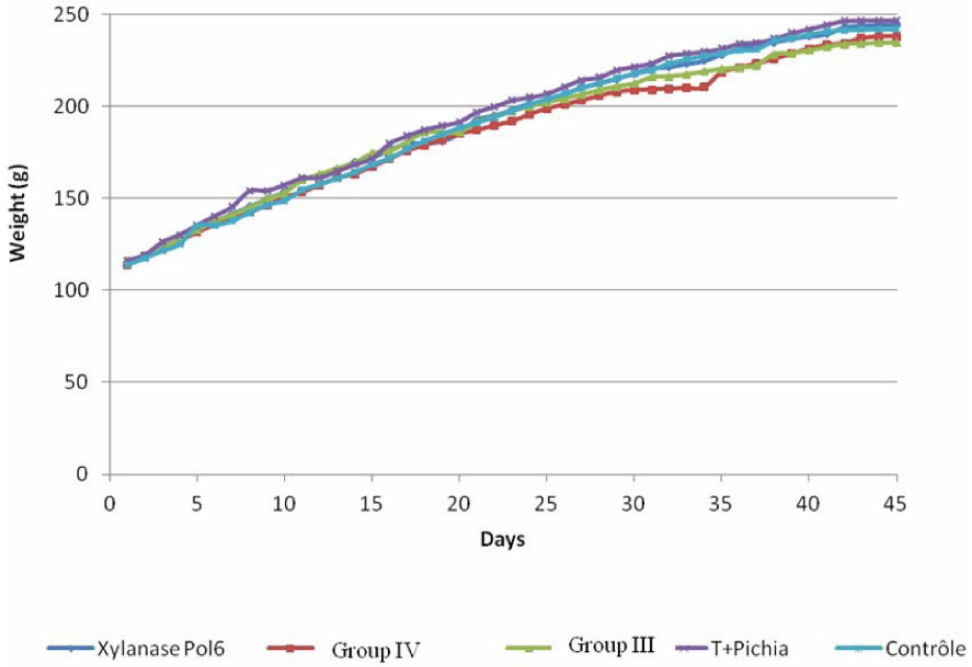

Figure 1 : Development in body weight of the controls and treated groups in adult rats during 45 days.

TABLE 4 : Relative organes weight of kidney, liver, heart and spleen in control and treated rats.

Groups	Relative organes weight (g/100 g body weight)			
	kidney	liver	heart	spleen
Group I	0.45 ±0.04	4.94 ± 1.08	0.40 ± 0.03	0.34 ± 0.06
Groupe II	0.45 ± 0.07	4.59 ± 0.91	0.44 ± 0.08	0.34 ± 0.08
Groupe III	0.48 ± 0.06	4.75 ± 1.03	0.43 ± 0.04	0.33 ± 0.03
Groupe IV	0.45 ± 0.03	5.13 ± 0.89	0.42 ± 0.04	0.31 ± 0.04
Groupe V	0.45 ± 0.04	4.92 ±1.00	0.43 ± 0.07	0.35 ± 0.08

Values are means+SD for seven rats in each group.

the control group, indicating the absence of activation of defense mechanism (TABLE 5).

Creatinine and urea levels in plasma and urine

Our results showed a normal renal function of the treated group objectified by normal values of Creatinine levels in the plasma and urine when compared to controls. Urea levels in plasma and in urine were unchanged in treated rats when compared to those of controls (TABLE 6).

Estimation of lipid peroxidation

Our results revealed that plasma MDA levels of treated groups were unchanged when compared to controls (Figure 2).

Markers of protein oxidative damage

Figure 2 showed plasma levels of protein carbonyls (PCO), indice of protein oxidative damage, in normal and experimental animals. In treated groups no significant changes of plasma PCO levels were observed when compared to those of controls.

Plasma enzymatic and non-enzymatic antioxidants

In treated rats, plasma superoxide dismutase and catalase activities and GSH levels didn't change when

TABLE 5 : Hematological parameters of in control and treated rats.

Hematological parameters	Group I	Group II	Group III	Group VI	Group V
GB (10^3/µl)	11.64 ±1.37	11.37 ±0.34	10.96 ±1.20	11.59 ±0.45	11.4 ±0.75
GR (10^6/µl)	8.15 ±1.63	7.58 ±0.80	9.54 ±0.94	8 ±0.90	8.25 ±0.77
Hb (g/dl)	14.2 ±2.71	13.37 ±1.49	15.71 ±1.67	13.91 ±1.00	13 ±4.81
Ht (%)	43.44 ±9.21	41.13 ±4.73	49.01 ±6.15	42.6 ±4.16	44.85 ±4.04
MCV (mm^3/GR)	53.21 ±1.66	54.24 ±1.38	53.29 ±1.34	53.33 ±1.37	54.38 ±1.48
MCH (pg/GR)	17.47 ±0.55	17.63 ±0.44	17.11 ±0.35	17.47 ±0.76	15.53 ±5.38
MCHC (g/dl)	32.86 ±0.93	32.53 ±0.45	32.16 ±0.88	32.74 ±0.95	28.57 ±9.84

Les valeurs sont exprimées en moyenne ± l'écart type ; n= 7 rats

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TABLE 6 : Biochemical markers in plasma

Parameters & treatments		Group I	Group II	Group III	Group IV	Group V
Urea [α]	Plasma	19.44± 6.804	22.222±8.606	19.444± 6.804	22.222±8.606	22.222±8.606
	Urine	100± 61.237	108. 333±58.452	100± 50	83.333±28.867	87.500 ±25
Creatinine [β]	Plasma	78.2± 0.1222	56.4±0.310	79.1± 0.323	64.6±0.198	74.1±0.107
	Urine	67.272± 14.590	72±33.150	40.727± 4.741	44.727±5.763	80 ±56.836
Albumine [γ]		34.28± 1.63	34.05±1.73	33.80± 0.76	33.68±1.28	34.23 ±0.66

Values are expressed as means ± S.D for seven animals in each group; [α] Urea: mmoles/l.; [β] Creatinine: µmoles/l.; [γ] Albumine: g/l.

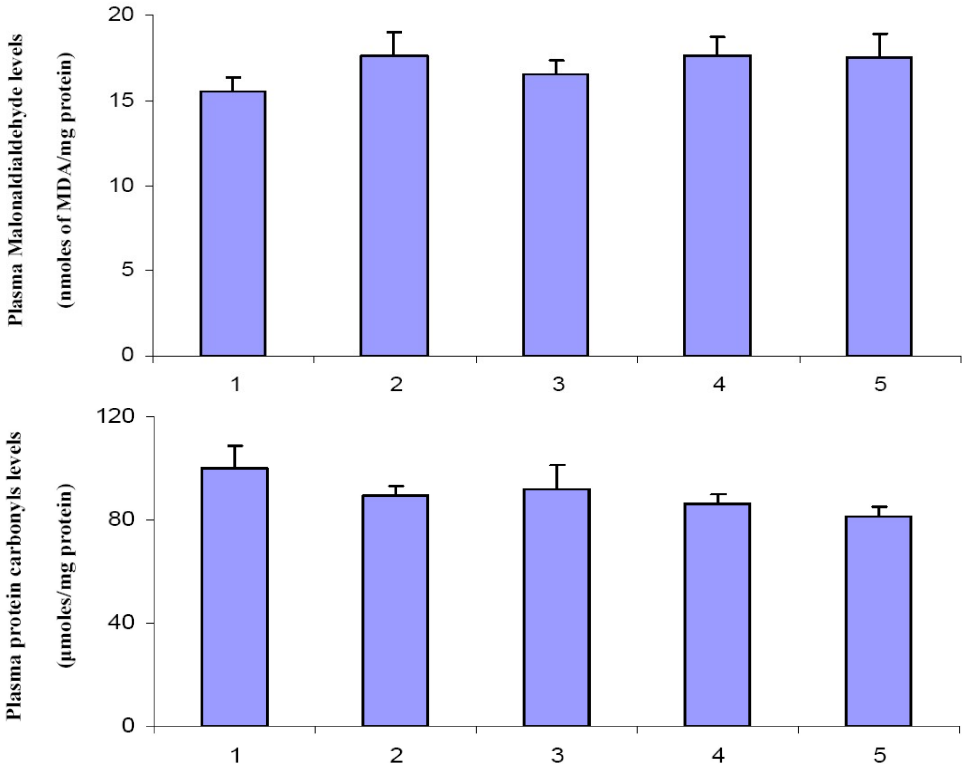

Figure 2 : Plasma levels of both malondialdehyde protein (MDA) and protein carbonyls (PCO) in controls and treated rats.

compared to those of controls (TABLE 7). The hydrophilic antioxidant levels (vitamin C) in plasma of treated rats were not modified when compared to controls.

Histological studies

In the treated rats, kidney histological studies showed no abnormalities (Figure 3) detected in glomeruli and in convoluted tubules, when compared to controls.

Bacterial reverse mutation assay

The results of these experiments are presented in TABLE 8. The sensitivity of the individual bacterial strains and the metabolising potential of the S9 mix was confirmed by significant increases in the number of revertant colonies by diagnostic mutagens. Based on the

TABLE 7 : Enzymatic antioxidant activities (glutathione peroxidase and catalase) in the plasma of adult rat controls or treated during 41 days.

Parameters & treatments	Group I	Group II	Group III	Group IV	Group V
Catalase [α]	53.339 ±5.579	50.722± 9.083	51.034 ±9.545	53.247 ±4.255	53.025 ± 7. 411
Superoxide dismutase [β]	257.381 ± 15.199	259.979 ± 7.136	261.642 ± 48.473	253.588 ± 24.120	254.443 ± 25.566
Glutathione [γ]	43.065±3.395	42.736± 5.975	42.497±2.206	37.026±2.190	36.402±3.030
Vitamin C [£]	33.571±2.520	33.773± 2.271	34.255±2.166	34.227±1.906	37.426±3.240

Values are expressed as means ± S.D for seven animals in each group; [α] Catalase: µmoles H_2O_2 consumed/min/mg of protein.; [β] Superoxide dismutase: units/mg of protein; [γ] Glutathione: µg/mg of protein; £ vitamin C: µmoles of ascorbic acid/mg protein.

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viable counts as well as microscopic examination of the bacterial background lawns, no toxicity or inhibition of growth was observed at any dose level of the test substance. In fact distinct growth stimulation was observed in several of the test series, however without affecting the results of the study. No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to the expressed xylanase either in the presence or absence of S9 mix.

DISCUSSION

For The fungus *P.occitanis* Pol6, no knowledge in the literature was indicated of its toxigenic effect. While, the results from our study confirmed that the xylanase enzyme preparation of this microorganism does not produce toxicity neither *in vivo* nor *in vitro*.

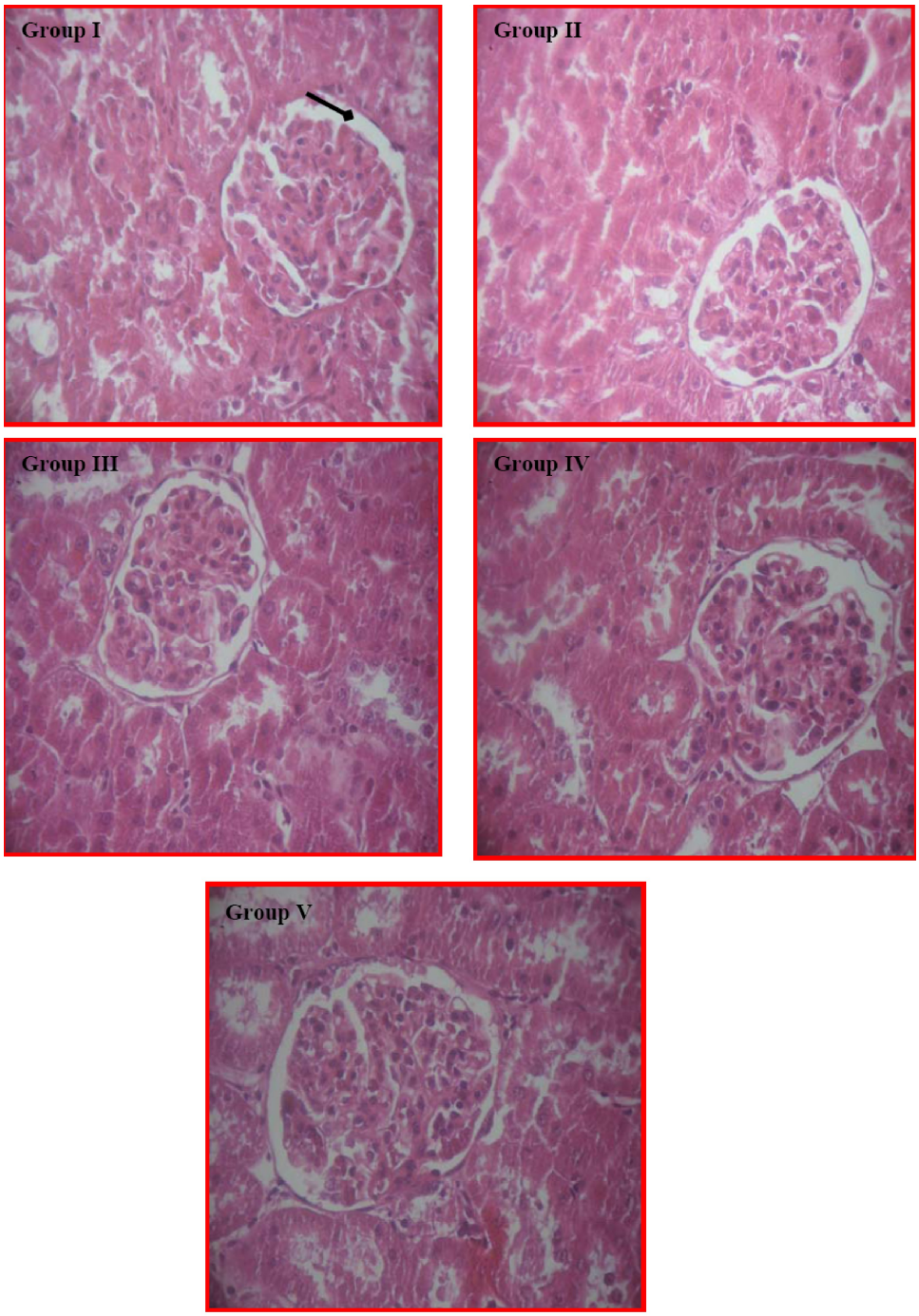

Figure 3 : Histological kidney sections in controls and treated rats via their drinking water. Opticmicroscopy: (hematoxylin and eosin 400X). Arrows indicate:→ Bowman's space.

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TABLE 8 : Bacterial reverse mutation assay. Mean number of revertant colonies per plate.

Treatment µg/ml	S. typhimurium					E. coli
	S9	TA 1537	TA 98	TA 1535	TA 100	WP2uvrA pkM101
1. Experiment						
expressed xylanases						
2500	_	16	22	11	82	219
1500	_	16	17	11	85	192
156	_	13	19	8	95	188
Solvent	_	473				
9-AA (2 mg)	_		891			
2-NF (20 mg)	_			2801		
MNNG (1 mg)	_				3332	
MNNG (7.5 mg)	_					1066
expressed xylanases						
2500	+	16	26	13	114	278
1500	+	14	25	12	101	256
156	+	10	21	11	95	276
Solvent	+	162	2356	174	2121	
2-AA (5 mg)	+					1242
2-AA (20 mg)	+					
2. Experiment						
expressed xylanases						
2500	_	14	26	16	126	165
1500	_	8	23	14	105	164
156	_	12	17	13	93	154
Solvent	_	368				
9-AA (2 mg)	_		1017			
2-NF (20 mg)	_			2014	2615	
MNNG (1 mg)	_					
MNNG (7.5 mg)	_					1233
expressed xylanases						
2500	+	11	26	9	81	375
1500	+	11	24	12	92	278
156	+	14	33	11	95	276
Solvent	+	190	2063	144	2092	
2-AA (5 mg)	+					897
2-AA (20 mg)	+					

Repeated experiment Treatment µg/mL	E.coli WP2 uvrA pkM101			
	S9	Revertants		viable count
		With tryptophan	Without tryptophan	
expressed xylanases				
2500	+	284	157	678
1500	+	273	124	564
156	+	266	127	270
Solvent	+	212	88	304
2-AA (20 mg)	+	1534		

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According to[6], *P. pastoris* has been safely used for the production of over 300 recombinant proteins since the mid-1980s and fulfills the criteria of several safety evaluations. Many human genes have been expressed in *P. pastoris* for pharmaceutical use. In addition, *P. Pastoris* itself is approved by FDA as a source of animal feed protein for use in broiler feed up to 10% of the total feed[16]. Toxicity studies and oxidative stress[16] undertaken as a support of *P. pastoris*-approved animal feed (including a pathogenicity study in mice, an acute or a subacute oral toxicity study in rats) also demonstrated that *P. pastoris* is neither pathogenic nor toxigenic

In general, oxidative stress is a constant threat to all living organisms and endogenous antioxidant defense system is employed by the body to its elimination or attenuation[5].

Erythrocytes are very sensitive to oxidative injury[4]. Extensive lipid peroxidation causes changes in fluidity and an increase in the permeability to different ions leading to hemolysis. To defend themselves against oxidative stress, erythrocytes are equipped with an effective and complexe antioxidant system, including protective enzymes and biological antioxidants such as SOD, CAT, GPx, GSH and vitamins C and E. In our current study, we aimed to recognize if administration of xylanases to adult rats caused toxicity.

In fact, the exposure of adult rats to xylanases during 45 days did not affect their hematological parameters. Red blood cells number, hematocrit values and hemoglobin concentration revealed no significant changes in treated groups when compared to those of controls. Other hematological parameters, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, remained also unchanged in adult treated rats. We also found that xylanases didn't affect the immune function of the treated groups. Indeed, white blood cells number remained unchanged in treated rats, indicating that xylanases didn't activate the immune defense system.

It is well known that vitamin C (ascorbic acid) is among the most important antioxidants which influences the immune system. Vitamin C is a potent antioxidant that offers protection against oxidative damage to various tissues. In the current study, plasma vitamins C level remained unchanged after treatment with xylanases. These results spoke in favor of immune dysfunction in treated rats.

In addition to hematological parameters, some plasma biochemical parameters were assayed. Different mechanisms have been postulated to explain toxicity of several xenobiotics, such as lipid peroxidation and interaction with membrane components. In fact, lipid peroxidation (LPO) represents one of the most frequent reactions resulting from free radicals' attack on biological structures. In our study, the plasma malondialdehyde level, indicator of lipid peroxidation, remained unaffected in xylanases treated rats. The latters didn't induce LPO or physiological and biochemical changes of biological systems as demonstrated by our results. So, the absence of LPO in the plasma suggested the safety use of xylanases because they didn't produce free-radicals and oxidative injury to protein, the mediating factor of the toxicity. In view of our results, we suggest that exposure to xylanases did not cause toxicity. Thus, xylanases might be safety used in food processing.

Plasma antioxidants are the agents involved in the defence against reactive oxygen species that are used up most rapidly. Antioxidant enzymes such as superoxide dismutase and catalase, constitute mutually a supportive team of defence against reactive oxygen species. Our results showed that the activities of these enzymes were not affected in treated rats when compared to those of controls. Likewise, glutathione, a crucial component of the antioxidant defence mechanism, functions as a direct reactive free-radical scavenger. Glutathione levels were also unchanged in treated groups, indicating the safety use of xylanases in food industries. It is well known that the kidney is the critical target organ for xenobiotic compounds which produce a variety of renal toxic effects involving tubular cells and glomerulus[25].

Twenty four hours urine volumes are usually studied as indices of the functional integrity of renal proximal tubules, while proper glomerular function is assessed by examining the plasma and urinary creatinine and urea levels.

Creatinemia is a more potent indicator than urea in the first phases of kidney function. In the current work, the unchanged plasma urea and creatinine levels of treated groups reflected a normal renal function. To substantiate the biochemical findings, a histological examination of the kidney was undertaken. In fact, histological pictures seen in the kidneys of treated rats were

characterized by a normal histoarchitecture.

Another biomarker of toxicity was also studied. In fact, albumin synthetised by the liver is excreted in the plasma, was not changed indicating no inflammatory reactions. Thus confirmed the unchanged white blood cells number. We concluded that xylanases from both strains didn't affect the immune function.

Based on the bacterial reverse mutation assay data it is concluded that the expressed xylanase in *P. pastoris* do not present mutagenic or clastogenic potential when tested in relevant genotoxicological assays.

The adverse effects from toxicology studies could be used in the calculation of safety margins based on the enzyme's applications, the use levels of the enzyme in the application and human consumption data. A theoretical calculation of human exposure to the xylanase through consumption of bread can be made on the following assumptions: the consumption of bakery products is estimated to be 200 g/person/day[15,26] USDA, ARS, 1997;). The flour content in bakery products is estimated to be 66% and for a body weight of 70 kg the intake of flour from bakery products is then: 2g flour/ kg body weight/day. The maximum dose of xylanase to be expected in bakery products is 4000 TXU/kg flour. Therefore, the maximum estimated daily intake (EDI) of xylanase through bakery products is 400 TXU/kg body weight/day. The maximum dose used in 45 days of experimental study in rats was 12000 TXU/kg body weight/day giving a no observed- effect level (NOEL) of 12000 TXU/kg/day. Thus the safety margin is: NOEL/EDI=30.

Studies of these xylanase products have shown an important safety margin of 30 based on a relatively high expected dose of the xylanase in food, this safety margin is lower than that calculated for the xylanase from *Bacillus subtilis*[19]. The safety of these xylanases is further supported by the active approval of both *Pichia pastoris* as a production organism and of xylanase for the use in food and feed.

CONCLUSION

This paper constitutes the first report which demonstrates the safety use of xylanases from *P. occitanis* Pol6 and the expressed ones in *P. pastoris*. In fact, all the data presented in this work establish that the studied xylanases possess no discernible risk to human health and could be applied in the food industry. Other studies will be needed to demonstrate the last word on safety. In this way, further biochemical and molecular studies would be one of logical next steps in order to check the non toxicity of xylanases.

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