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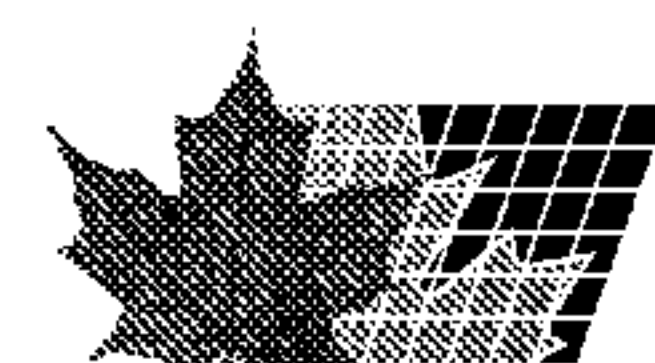
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(57) **Abrégé/Abstract:**

Provided are dietary aids for delivery of digestive enzymes, therapeutics and agents to an organism. The dietary aids are designed for localization to the gizzard of an organism and provide sustained release of an active agent within the digestive tract of the organism.



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DIETARY AIDS AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

5 The present invention is generally related to dietary aids and more specifically to compositions and methods for delivery of digestive enzymes, therapeutics and agents to a subject.

BACKGROUND

10 Proper enzymatic digestion or breakdown of foods and minerals in the digestive tract of organisms is critical for health and growth. For example, minerals are essential elements for the growth of all organisms. For livestock production of monogastric animals (*e.g.*, pigs, poultry) and fish, feed is commonly supplemented with minerals. Plant seeds are a rich source of minerals since they
15 contain ions that are complexed with the phosphate groups of phytic acid. Ruminants do not require inorganic phosphate and minerals because microorganisms in the rumen produce enzymes that catalyze conversion of phytate (myo-inositol-hexaphosphate) to inositol and inorganic phosphate. In the process, minerals that have been complexed with phytate are released.

20 Phytate occurs as a source of stored phosphorous in virtually all plant feeds (Phytic Acid, Chemistry and Applications, E. Graf (Ed.), Pilatus Press: Minneapolis, Minn., U.S.A., 1986). Phytic acid forms a normal part of the seed in cereals and legumes and binds dietary minerals that are essential to the new plant
25 as it emerges from the seed. When the phosphate groups of phytic acid are removed by the seed enzyme phytase, the ability to bind metal ions is lost and the minerals become available to the plant. In livestock feed grains, the trace minerals bound by phytic acid are only partially available for absorption by monogastric animals, which lack phytase activity. Although some hydrolysis of phytate occurs
30 in the colon, most phytate passes through the gastrointestinal tract of monogastric animals and is excreted in the manure contributing to fecal phosphate pollution problems in areas of intense livestock production. Inorganic phosphorous released

in the colon has no nutritional value to livestock because inorganic phosphorous is absorbed only in the small intestine. Thus, a significant amount of the nutritionally important dietary minerals are potentially unavailable to monogastric animals.

5 Conversion of phytate to inositol and inorganic phosphorous can be catalyzed by microbial enzymes referred to broadly as phytases. Phytases such as phytase #EC 3.1.3.8 are capable of catalyzing hydrolysis of myo-inositol hexaphosphate to D-myo-inositol 1,2,4,5,6-pentaphosphate and orthophosphate. Certain fungal phytases reportedly hydrolyze inositol pentaphosphate to tetra-, tri-
10 , and lower phosphates; *e.g.*, *A. ficuum* phytases reportedly produce mixtures of myoinositol di- and mono-phosphate (Ullah, 1988). Phytase producing microorganisms comprise bacteria such as *Bacillus subtilis* (V. K. Powar and V. J. Jagannathan, J. Bacteriol. 151:1102-1108, 1982) and *Pseudomonas* (D. J. Cosgrove, Austral. J. Biol. Sci. 2:1207-1220, 1970); yeast such as *Sacchoromyces*
15 *cerevisiae* (N. R. Nayini and P. Markakis, Lebensmittel Wissenschaft und Technologie 17:24-26, 1984); and fungi such as *Aspergillus terreus* (K. Yamada, *et al.*, Agric. Biol Chem. 32:1275-1282, 1968). The possible use of microbes capable of producing phytase as a feed additive for monogastric animals has been reported previously (Shieh and Ware, U.S. Pat. No. 3,297,548; Nelson, T. S. *et al.*,
20 J. Nutrition 101:1289-1294, 1971).

 Microbial phytases may also reportedly be useful for producing animal feed from certain industrial processes, *e.g.*, wheat and corn waste products. The wet milling process of corn produces glutens sold as animal feeds. Addition of
25 phytase may reportedly improve the nutritional value of the feed product. Fungal phytase enzymes and process conditions are about 50 °C and a pH of about 5.5 as reported previously in European Patent Application 0 321 004. In processing soybean meal the presence of phytate reportedly renders the meal and wastes unsuitable for feeds used in rearing fish, poultry and other non-ruminants as well
30 as calves fed on milk. Phytase is reportedly useful for improving the nutrient and commercial value of this high protein soy material (see Finase Enzymes by Alko, Rajamaki, Finland). A combination of phytase and a pH 2.5 optimum acid

phosphatase from *A. niger* has been used by Alko, Ltd as an animal feed supplement in their phytic acid degradative product Finas F and Finase S. A cost-effective source of phytase would greatly enhance the value of soybean meals as an animal feed (Shieh *et al.*, 1969).

5

Phytase and less specific acid phosphatases are produced by the fungus *Aspergillus ficuum* as extracellular enzymes (Shieh *et al.*, 1969). Ullah reportedly purified a phytase from wild-type *A. ficuum* that had an apparent molecular weight of 61.7 kDA (on SDS-PAGE; as corrected for glycosylation); pH optima at pH 2.5 and pH 5.5; a K_m of about 40 μM ; and, a specific activity of about 50 U/mg (Ullah, A., Preparative Biochem 18:443-458, 1988); PCT application WO 91/05053 also reportedly discloses isolation and molecular cloning of a phytase from *Aspergillus ficuum* with pH optima at pH 2.5 and pH 5.5, a K_m of about 250 μm , and specific activity of about 100 U/mg protein.

15

Accordingly, regulating and affecting digestion of foods is important to the growth and development an organism.

SUMMARY OF THE INVENTION

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The invention provides a dietary aid release vehicle characterized as a biocompatible composition comprising an agent that assists in digestion, wherein the biocompatible composition is designed for oral consumption and release in the digestive tract of a subject.

25

In another embodiment, the invention provides a method of aiding digestion of an organism by delivering to the digestive tract of the organism a biocompatible composition for release of an agent that assists in digestion upon oral consumption, wherein release of the agent results in aiding digestion of the organism.

30

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

5

FIGURE 1a-1c show a nucleotide and deduced amino acid sequences an enzyme useful in the compositions of the invention (SEQ ID Nos:1 and 2, respectively).

10

FIGURE 2 shows the pH and temperature profile and stability data for a phytase enzyme. The assay used for these analysis is the following for the detection of phytase activity: Phytase activity is measured by incubating 150 μ l of the enzyme preparation with 600 μ l of 2 mM sodium phytate in 100 mM Tris HCl buffer pH 7.5, supplemented with 1 mM CaCl₂ for 30 minutes at 37 °C. After incubation the reaction is stopped by adding 750 μ l of 5% trichloroacetic acid. Phosphate released was measured against phosphate standard spectrophotometrically at 700 nm after adding 1500 μ l of the color reagent (4 volumes of 1.5% ammonium molybdate in 5.5% sulfuric acid and 1 volume of 2.7% ferrous sulfate; Shimizu, M., 1992; Biosci. Biotech. Biochem., 56:1266-1269). OD at 700 nm is indicated on the Y-axis of the graphs in FIG. 2. Temperature or pH is indicated on the X-axis of the graphs.

15

20

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a digestive aid containing an enzyme either as the sole active ingredient or in combination with one or more other agents and/or enzymes. The use of enzymes and other agents in digestive aids of livestock or domesticated animals not only improves the animal's health and life expectancy but also assists in increasing the health of livestock and in the production of foodstuffs from livestock.

25

30

Currently, some types of feed for livestock (*e.g.*, certain poultry feed) are highly supplemented with numerous minerals (*e.g.*, inorganic phosphorous),

enzymes, growth factors, drugs, and other agents for delivery to the livestock. These supplements replace many of the calories and natural nutrients present in grain.

5 By reducing or eliminating the inorganic phosphorous supplement and other supplements (*e.g.*, trace mineral salts, growth factors, enzymes, antibiotics) from the feed itself, the feed would be able to carry more nutrient and energy. Accordingly, the remaining diet would contain more usable energy. For example, grain-oilseed meal diets generally contain about 3,200 kcal metabolizable energy
10 per kilogram of diet, and mineral salts supply no metabolizable energy. Removal of the unneeded minerals and substitution with grain would therefore increase the usable energy in the diet. Thus, the invention can be differentiated over commonly used phytase containing feed. For example, in one embodiment, a biocompatible material is used that is resistant to digestion by the gastrointestinal
15 tract of an organism.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the
20 laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (*e.g.*, electroporation, lipofection). Generally, enzymatic reactions and purification steps are performed
25 according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook *et al.* Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this
30 document.

As used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus,

for example, reference to “an enzyme” includes a plurality of enzymes and reference to “the agent” generally includes reference to one or more agents and equivalents thereof known to those skilled in the art, and so forth.

All publications mentioned herein are incorporated herein by reference in full
5 for the purpose of describing and disclosing the databases, proteins, and
methodologies, which are described in the publications which might be used in
connection with the presently described invention. The publications discussed above
and throughout the text are provided solely for their disclosure prior to the filing date
of the present application. Nothing herein is to be construed as an admission that the
10 inventors are not entitled to antedate such disclosure by virtue of prior invention.

The headings and subheadings used herein are for the convenience of the
reader and are not intended to limit the invention.

In many organisms, including, for example, poultry or birds such as, for
example, chickens, turkeys, geese, ducks, parrots, peacocks, ostriches, pheasants,
15 quail, pigeons, and dove, the digestive tract includes a gizzard which stores and
uses hard biocompatible objects (*e.g.*, rocks) to help in the digestion of seeds or
other feed consumed by a bird. A typical digestive tract of this general family of
organisms, includes the esophagus which contains a pouch, called a crop, where
food is stored for a brief period of time. From the crop, food moves down into the
20 true stomach, or *proventriculus*, where hydrochloric acid and pepsin start the
process of digestion. Next, food moves into the gizzard, which is oval shaped and
thick walled with powerful muscles. The chief function of the gizzard is to grind
or crush food particles - a process which is aided by the bird swallowing small
amounts of fine gravel or grit. From the gizzard, food moves into the duodenum.
25 The small intestine of birds is similar to mammals. There are two blind pouches
or *ceca*, about 4-6 inches in length at the junction of the small and large intestine.
The large intestine is short, consisting mostly of the rectum about 3-4 inches in
length. The rectum empties into the *cloaca* and feces are excreted through the
vent.

Hard, biocompatible objects consumed (or otherwise introduced) and presented in the gizzard provide a useful vector for delivery of various enzymatic, chemical, therapeutic and antibiotic agents. These hard substances have a life span of a few hours to a few days and are passed after a period of time.

5 Accordingly, the invention provides coated, impregnated (*e.g.*, impregnated matrix and membranes) modified dietary aids for delivery of useful digestive or therapeutic agents to an organism. Such dietary aids include objects which are typically ingested by an organism to assist in digestion within the gizzard (*e.g.*, rocks or grit). The invention provides biocompatible objects that have coated
10 thereon or impregnated therein agents useful as a digestive aid for an organism or for the delivery of a therapeutic or medicinal agent or chemical.

In a first embodiment, the invention provides a dietary aid, having a biocompatible composition designed for slow release of an agent that assists in
15 digestion, wherein the biocompatible composition is designed for oral consumption and release in the gizzard of an organism. "Biocompatible" means that the substance, upon contact with a host organism (*e.g.*, a bird), does not elicit a detrimental response sufficient to result in the rejection of the substance or to render the substance inoperable. Such inoperability may occur, for example, by
20 formation of a fibrotic structure around the substance limiting diffusion of impregnated agents to the host organism therein or a substance which results in an increase in mortality or morbidity in the organisms due to toxicity or infection. A biocompatible substance may be non-biodegradable or biodegradable. In one
25 embodiment, the biocompatible composition is resistant to degradation or digestion by the gastrointestinal tract. In another embodiment, the biocompatible composition has the consistency of a rock or stone.

A non-biodegradable material useful in the invention is one that allows attachment or impregnation of a dietary agent. Such non-biodegradable materials
30 include, for example, thermoplastics, such as acrylic, modacrylic, polyamide, polycarbonate, polyester, polyethylene, polypropylene, polystyrene, polysulfone, polyethersulfone, and polyvinylidene fluoride. Elastomers are also useful

materials and include, for example, polyamide, polyester, polyethylene, polypropylene, polystyrene, polyurethane, polyvinyl alcohol and silicone (*e.g.*, silicone based or containing silica). The invention provides that the biocompatible composition can contain a plurality of such materials, which can be, *e.g.*, admixed
5 or layered to form blends, copolymers or combinations thereof.

As used herein, a “biodegradable” material means that the composition will erode or degrade *in vivo* to form smaller chemical species. Degradation may occur, for example, by enzymatic, chemical or physical processes. Suitable
10 biodegradable materials contemplated for use in the invention include poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides, polycarbonate, polycyanoacrylate, polyurethanes, polyacrylate. Such materials can be admixed or layered to form blends, copolymers or
15 combinations thereof.

It is contemplated that a number different biocompatible substances may be ingested or otherwise provided to the same organism simultaneously, or in various combinations (*e.g.*, one material before the other). In addition, the
20 biocompatible substance may be designed for slow passage through the digestive tract. For example, large or fatty substances tend to move more slowly through the digestive tract, accordingly, a biocompatible material having a large size to prevent rapid passing in the digestive tract can be used. Such large substances can be a combination of non-biodegradable and biodegradable substances. For
25 example, a small non-biodegradable substance can be encompassed by a biodegradable substance such that over a period of time the biodegradable portion will be degraded allowing the non-biodegradable portion to pass through the digestive trace. In addition, it is recognized that any number of flavorings or appearance enhancers can be provided to the biocompatible substance to assist in
30 consumption (*e.g.*, ligands for taste receptors or smell receptors).

Any number of agents alone or in combination with other agents can be coated on the biocompatible substance including polypeptides (*e.g.*, enzymes, antibodies, cytokines or therapeutic small molecules), and antibiotics, for example. Examples of particular useful agents are listed in Table 1 and 2, below.

- 5 It is also contemplated that cells can be encapsulated into the biocompatible material of the invention and used to deliver the enzymes or therapeutics. For example, porous substances can be designed that have pores large enough for cells to grow in and through and that these porous materials can then be taken into the digestive tract. For example, the biocompatible substance can be comprised of a
- 10 plurality of microfloral environments (*e.g.*, different porosity, pH etc.) that provide support for a plurality of cell types. The cells can be genetically engineered to deliver a particular drug, enzyme or chemical to the organism. The cells can be eukaryotic or prokaryotic.

15

TABLE 1

Treatment Class	Chemical	Description
Antibiotics	Amoxycillin and Its Combination Mastox Injection (Amoxycillin and Cloxacillin)	Treatment Against Bacterial Diseases Caused By Gram + and Gram – Bacteria
	Ampicillin and Its Combination BioloX Injection (Ampicillin and Cloxacillin)	Treatment Against Bacterial Diseases Caused By Gram + And Gram – Bacteria.
	Nitrofurazone + Urea Nefrea Bolus	Treatment Of Genital Infections
	Trimethoprim + Sulphamethoxazole Trizol Bolus	Treatment Of Respiratory Tract Infections, Gastro Intestinal Tract Infections, Urino- Genital Infections.
	Metronidazole and Furazolidone Metofur Bolus	Treatment Of Bacterial And Protozoal Diseases.
	Phthalylsulphathiazole, Pectin and Kaolin Pectolin Bolus Suspension	Treatment Of Bacterial And Non-Specific Diarrhoea, Bacillary Dysentery And Calf Scours.
Antihelmintics	Ectoparasiticide Germex Ointment (Gamma Benzene Hexachloride, Proflavin Hemisulphate and Cetrinide)	Ectoparasiticide and Antiseptic
	Endoparasiticide > Albendazole and Its Combination Alben (Albendazole) Suspension (Albendazole 2.5%) Plus Suspension (Albendazole	Prevention And Treatment Of Roundworm, Tapeworm and Fluke Infestations

	Alpraz (Albendazole and Praziquantel)Tablet	Prevention And Treatment Of Roundworm and Tapeworm Infestation In Canines and Felines.
	Oxyclozanide and Its Combination Clozan (Oxyclozanide) Bolus, Suspension	Prevention and Treatment Of Fluke Infestations
	Tetzan (Oxyclozanide and Tetramisole Hcl) Bolus, Suspension	Prevention and Treatment Of Roundworm and Fluke Infestations
	Fluzan (Oxyclozanide and Levamisole Hcl) Bolus, Suspension	Prevention and Treatment Of Roundworm Infestations and Increasing Immunity
	Levamisole Nemasol Injection Wormnil Powder	Prevention and Treatment Of Roundworm Infestations and Increasing Immunity.
	Fenbendazole Fenzole Tablet (Fenbendazole150 Mg.) Bolus (Fenbendazole 1.5 Gm.) Powder (Fenbendazole 2.5% W/W)	Prevention And Treatment of Roundworm and Tapeworm Infestations
Tonics	Vitamin B Complex, Amino Acids and Liver Extract Heptogen Injection	Treatment Of Anorexia, Hepatitis, Debility, Neuralgic Convulsions Emaciation and Stunted Growth.
	Calcium Levulinate With Vit.B ₁₂ and Vit D ₃ Hylactin Injection	Prevention and treatment of hypocalcaemia, supportive therapy in sick conditions (especially hypothermia) and treatment of early stages of rickets.
Animal Feed Supplements	Essential Minerals, Selenium and Vitamin E Gynolactin Bolus	Treatment Of Anoestrus Causing Infertility and Repeat Breeding In Dairy Animals and Horses.
	Essential Minerals, Vitamin E, and Iodine Hylactin Powder	Infertility, Improper Lactation, Decreased Immunity, Stunted Growth and Debility.
	Essential Electrolytes With Vitamin C Electra – C Powder	Diarrhoea, Dehydration, Prior to and after Transportation, In Extreme temperatures (High Or Low) and other Conditions of stress.
	Pyrenox Plus (Diclofenac Sodium + Paracetamol) Bolus, Injection.	Treatment Of Mastitis, Pyrexia Post Surgical Pain and Inflammation, Prolapse Of Uterus, Lameness and Arthritis.

Table 2. Therapeutic Formulations

Product	Description
Acutrim[®] (phenylpropanolamine)	Once-daily appetite suppressant tablets.
The Baxter[®] Infusor	For controlled intravenous delivery of anticoagulants, antibiotics, chemotherapeutic agents, and other widely used drugs.
Catapres-TTS[®] (clonidine transdermal therapeutic system)	Once-weekly transdermal system for the treatment of hypertension.
Covera-HS[™] (verapamil hydrochloride)	Once-daily Controlled-Onset Extended-Release (COER-24) tablets for the treatment of hypertension and angina pectoris.

DynaCirc CR[®] (isradipine)	Once-daily extended release tablets for the treatment of hypertension.
Efidac 24[®] (chlorpheniramine maleate)	Once-daily extended release tablets for the relief of allergy symptoms.
Estraderm[®] (estradiol transdermal system)	Twice-weekly transdermal system for treating certain postmenopausal symptoms and preventing osteoporosis
Glucotrol XL[®] (glipizide)	Once-daily extended release tablets used as an adjunct to diet for the control of hyperglycemia in patients with non-insulin-dependent diabetes mellitus.
IVOMEC SR[®] Bolus (ivermectin)	Ruminal delivery system for season-long control of major internal and external parasites in cattle.
Minipress XL[®] (prazosin)	Once-daily extended release tablets for the treatment of hypertension.
NicoDerm[®] CQ[™] (nicotine transdermal system)	Transdermal system used as a once-daily aid to smoking cessation for relief of nicotine withdrawal symptoms.
Procardia XL[®] (nifedipine)	Once-daily extended release tablets for the treatment of angina and hypertension.
Sudafed[®] 24 Hour (pseudoephedrine)	Once-daily nasal decongestant for relief of colds, sinusitis, hay fever and other respiratory allergies.
Transderm-Nitro[®] (nitroglycerin transdermal system)	Once-daily transdermal system for the prevention of angina pectoris due to coronary artery disease.
Transderm Scop[®] (scopolamin transdermal system)	Transdermal system for the prevention of nausea and vomiting associated with motion sickness.
Volmax (albuterol)	Extended release tablets for relief of bronchospasm in patients with reversible obstructive airway disease.
Actisite[®]	(tetracycline hydrochloride) Periodontal fiber used as an adjunct to scaling and root planing for reduction of pocket depth and bleeding on probing in patients with adult periodontitis.
ALZET[®]	Osmotic pumps for laboratory research.
Amphotec[®] (amphotericin B cholesteryl sulfate complex for injection)	AMPHOTEC [®] is a fungicidal treatment for invasive aspergillosis in patients where renal impairment or unacceptable toxicity precludes use of amphotericin B in effective doses and in patients with invasive aspergillosis where prior amphotericin B therapy has failed.
BiCitra[®] (sodium citrate and citric acid)	Alkalinizing agent used in those conditions where long-term maintenance of alkaline urine is desirable.
Ditropan[®] (oxybutynin chloride)	For the relief of symptoms of bladder instability associated with uninhibited neurogenic or reflex neurogenic bladder (<i>i.e.</i> , urgency, frequency, urinary leakage, urge incontinence, dysuria).
Ditropan[®] XL (oxybutynin chloride)	is a once-daily controlled-release tablet indicated for the treatment of overactive bladder with symptoms of urge urinary incontinence, urgency and frequency.
DOXIL[®] (doxorubicin HCl liposome injection)	
Duragesic[®] (fentanyl transdermal system) CII	72-hour transdermal system for management of chronic pain in patients who require continuous opioid analgesia for pain that cannot be managed by lesser means such as acetaminophen-opioid combinations, non-steroidal analgesics, or PRN dosing with short-acting opioids.
Elmiron[®] (pentosan polysulfate sodium)	Indicated for the relief of bladder pain or discomfort associated with interstitial cystitis.
ENACT AirWatch[™]	An asthma monitoring and management system.
Ethyol[®] (amifostine)	Indicated to reduce the cumulative renal toxicity associated with repeated administration of cisplatin in patients with advanced ovarian cancer or non-small cell lung cancer. Indicated to reduce the incidence of moderate to severe xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer, where the radiation port includes a substantial portion of the parotid glands.
Mycelex[®] Troche (clotrimazole)	For the local treatment of oropharyngeal candidiasis. Also indicated

	prophylactically to reduce the incidence of oropharyngeal candidiasis in patients immunocompromised by conditions that include chemotherapy, radiotherapy, or steroid therapy utilized in the treatment of leukemia, solid tumors, or renal transplantation.
Neutra-Phos[®] (potassium and sodium phosphate)	a dietary/nutritional supplement
PolyCitra[®] -K Oral Solution and PolyCitra[®] -K Crystals (potassium citrate and citric acid)	Alkalinizing agent useful in those conditions where long-term maintenance of an alkaline urine is desirable, such as in patents with uric acid and cystine calculi of the urinary tract, especially when the administration of sodium salts is undesirable or contraindicated
PolyCitra[®] -K Syrup and LC (tricitrates)	Alkalinizing agent useful in those conditions where long-term maintenance of an alkaline urine is desirable, such as in patients with uric acid and cystine calculi of the urinary tract.
Progestasert[®] (progesterone)	Intrauterine Progesterone Contraceptive System
Testoderm[®] Testoderm[®] with Adhesive and Testoderm[®] TTS CIII	Testosterone Transdermal System The Testoderm [®] products are indicated for replacement therapy in males for conditions associated with a deficiency or absence of endogenous testosterone: (1) Primary hypogonadism (congenital or acquired) or (2) Hypogonadotropic hypogonadism (congenital or acquired).
Viadur[™] (leuprolide acetate implant)	Once-yearly implant for the palliative treatment of prostate cancer

Certain agents can be designed to become active or in activated under certain conditions (*e.g.*, at certain pH's, in the presence of an activating agent etc.). In addition, it may be advantageous to use pro-enzymes in the compositions of the invention. For example, a pro-enzymes can be activated by a protease (*e.g.*, a salivary protease that is present in the digestive tract or is artificially introduced into the digestive tract of an organism). It is contemplated that the agents delivered by the biocompatible compositions of the invention can be activated or inactivated by the addition of an activating agent which may be ingested by, or otherwise delivered to, the organism. Another mechanism for control of the agent in the digestive tract is an environment sensitive agent that is activated in the proper digestive compartment. For example, an agent may be inactive at low pH but active at neutral pH. Accordingly, the agent would be inactive in the gut but active in the intestinal tract. Alternatively, the agent can become active in response to the presence of a microorganism specific factor (*e.g.*, microorganisms present in the intestine).

Enzymes are of particular relevance due to their role in digestion of foods consumed by the organism. For example, up to 80% of the phosphorus present in plant foods and feeds exists as a complex of phytic acid (myoinositol

hexaphosphate), hereinafter referred to as phytate (see co-pending U.S. Application 09/580,515, filed May 25, 2000, and PCT US00/14846, filed May 25, 2000 entitled "Recombinant Bacterial Phytase and Uses Thereof," both are incorporated herein by reference in their entirety). The phosphorus in phytate cannot be totally digested by simple-stomached animals, including humans, and it therefore passes through the gastrointestinal (GI) tract and is excreted in the feces. In animal nutrition, this is accounted for in diet formulation whereby 1.5 to 2.0% of an inorganic phosphate source is supplemented to meet the animal's minimal phosphate requirement. Addition of inorganic phosphorus to poultry, swine, dogs, cats, swine, bovine, and fish diets is expensive and is the third most expensive dietary ingredient, after energy and protein. The body requires phosphorus for formation of bones and teeth, for phospholipid (cell membrane structure) and nucleic acid (RNA, DNA) synthesis, for synthesis of ATP and other high-energy phosphate compounds, and for proper acid-base balance in the body. Roughly 85% of a body's phosphate is in the skeleton. Bone is comprised of 50% organic matrix (protein in the form of collagen, and lipid) and 50% inorganic material (mostly a calcium phosphate salt. *i.e.*, hydroxyapatite).

Supplemental inorganic phosphorous is provided to animal diets in one of three feedgrade forms; dicalcium phosphate (18.5% phosphorous), monocalcium phosphate (21.5% phosphorous) or deflorinated phosphate (18.0% phosphorous). In North America, 50% of feed-grade phosphate consumed is used for poultry feeding. It has been discovered that the use of the enzyme phytase in animal feed, could completely eliminate the need for supplemented inorganic phosphorous in animal feed.

In summary, the potential benefits of the present invention include, for example, (1) reduction in or possible elimination of the need for mineral supplements (*e.g.*, inorganic phosphorous supplements), enzymes, or therapeutic drugs for animal (including fish) from the daily feed or grain thereby increasing the amount of calories and nutrients present in the feed, and (2) increased health

and growth of domestic and non-domestic animals including, for example, poultry, porcine, bovine, equine, canine, and feline animals.

A large number of enzymes can be used in the methods and compositions of the present invention. These enzymes include enzymes necessary for proper digestion of consumed foods, or for proper metabolism, activation or derivation of chemicals, prodrugs or other agents or compounds delivered to the animal via the digestive tract. Examples of enzymes that can be delivered or incorporated into the compositions of the invention, include, for example, feed enhancing enzymes selected from the group consisting of α -galactosidases, β -galactosidases, in particular lactases, phytases, β -glucanases, in particular endo- β -1,4-glucanases and endo- β -1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4- β -galactosidases and arabinogalactan endo-1,3- β -galactosidases, endoglucanases, in particular endo-1,2- β -glucanase, endo-1,3- α -glucanase, and endo-1,3- β -glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan- α -rhamnosidase, pectate lyases, and α -galacturonisidases, mannanases, β -mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases. Phytases as set forth in SEQ ID NO:1 and 2 and in Table 3 below are preferred. The sequences described in Table 3 are SEQ ID NO:1 and 2 having the amino acid substitutions and nucleotide substitutions as described therein.

25

TABLE 3

Designation	Source	AA seq	Nuc. Sequence
E. coli B (reference)	E. coli B	S10; P26; D176; M298; A299; G312; I428	
868PH1	Bison E. coli	I428T	
872PH1	Kangaroo rat E. coli	D176G; G312S M298K; A299T	GAC(176)GGC; GGT(312)AG ATG(298)AAG; GCA(299)AC
875PH2	E. coli W	A160S; D176G; M298K; A299T	GCG(160)TCG; GAC(176)GG ATG(298)AAG; GCA(299)AC
873PH1	Calf E. coli	I428R	
E. coli B	E. coli B	K298M; T299A	AAG(298)ATG; ACA(299)GC
K12 appA	E. coli K12	M298K; A299T	ATG(298)AAG; GCA(299)AC

The enzymes used in the invention can be modified to enhance their activity, delivery, activation and degradation. Such modifications can be performed *in vivo* or *in vitro* and use methods and processes generally known in the art as described more fully below. Such methodology generally uses

5 polynucleotide or polypeptide sequences that are either synthesized by automated machines or are cloned, expressed, or manipulated by recombinant DNA techniques.

Polynucleotides

10 The invention provides polynucleotides encoding polypeptides having enzymatic activity for use in the compositions of the invention. The polynucleotides include recombinantly modified sequences as well as sequences encoding fusion polypeptides. In one embodiment, the polynucleotide of the invention encodes a phytase. A phytase can be derived from any number of organisms so long as the

15 polynucleotide encodes a polypeptide having phytase activity. Organisms useful as a source of phytase polypeptides and polynucleotides encoding phytase polypeptides include those listed in Table 3.

Table 3

Corynebacteria Corynebacterium diphtheria Pneumococci Diplococcus pneumoniae Streptococci Streptococcus pyrogenes Streptococcus salivarius Staphylococci Staphylococcus aureus Staphylococcus albus Neisseriae Neisseria meningitidis Neisseria gonorrhea Enterobacteriaceae Escherichia coli The colliform Aerobacter aerogenes bacteria Klebsiella pneumoniae Salmonella typhosa The Salmonellae

Salmonella choleraesuis
Salmonella typhimurium
Shigella dysenteria
The Shigellae
Shigella schmitzii
Shigella arabinotarda
Shigella flexneri
Shigella boydii
Shigella sonnei
Other enteric bacilli
Proteus vulgaris Proteus species
Proteus mirabilis
Proteus morgani
Pseudomonas aeruginosa
Alcaligenes faecalis
Vibrio cholerae
Memophilus-Bordetella group
Rhizopus oryzae
Hemophilus influenza, H. ducryi
Rhizopus arrhizua
Phycomycetes
Hemophilus hemophilus
Rhizopus nigricans
Hemophilus aegypticus
Sporotrichum schenkii
Hemophilus parainfluenzae
Flonsecaea pedrosoi
Bordetella pertussis
Flonsecaea compact
Pasteurellae Fonsecaea dermatidis
Pasteurella pestis
Cladosporium carrionii
Pasteurella tulareusis
Phialophora verrucosa
Brucellae Aspergillus nidulans
Brucella melitensis
Madurella mycetomi
Brucella abortus Madurella grisea
Brucella suis Allescheria boydii
Aerobic Spore-forming Bacilli
Phialophora jeanselmei
Bacillus anthracis
Microsporum gypseum
Bacillus subtilis Trichophyton mentagrophytes
Bacillus megaterium
Keratinomyces ajelloi
Bacillus cereus Microsporum canis
Anaerobic Spore-forming Bacilli
Trichophyton rubrum
Clostridium botulinum
Microsporum adouini
Clostridium tetani
Clostridium perfringens
Clostridium novyi Herpes Viruses
Clostridium septicum
Clostridium nistolyticum
Clostridium tertium
Clostridium bifermentans

Clostridium sporogenes
 Mycobacterium tuberculosis hominis
 Mycobacterium bovis
 Mycobacterium avium
 Mycobacterium leprae
 Mycobacterium paratuberculosis
 (fungus-like bacteria)
 Actinomyces Israeli
 Actinomyces bovis Cocksackievirus
 Actinomyces naeslundii
 Nocardia asteroides
 Nocardia brasiliensis
 The Spirochetes
 Treponema pallidum
 Spirillum minus
 Treponema pertenue
 Strepto- bacillus
 moniliformis
 Treponema carateum
 Borrelia recurrentis
 Leptospira icterohemorrhagiae
 Leptospira canicola
 Mycoplasma pneumoniae
 Listeria monocytogenes
 Erysipelothrix rhusiopathiae
 Streptobacillus moniliformis
 Donovanian granulomatis
 Bartonella bacilliformis
 Rickettsiae (bacteria-like parasites)
 Rickettsia prowazekii
 Rickettsia mooseri
 Rickettsia rickettsii
 Rickettsia conorii
 Rickettsia australis
 Rickettsia sibiricus
 Rickettsia akari Human Immunodeficiency
 Rickettsia tsutsugamushi
 Rickettsia burnetti
 Rickettsia quintana
 Chlamydia (unclassifiable parasites
 bacterial/viral)
 Chlamydia agents (naming uncertain)
 Fungi Rauscher
 Cryptococcus neoformans
 Blastomyces dermatidis
 Histoplasma capsulatum
 Coccidioides immitis
 Paracoccidioides brasiliensis
 Candida albicans
 Aspergillus fumigatus
 Mucor corymbifer (Absidia corymbifera)

The invention provides purified a recombinant enzyme that catalyzes the
 hydrolysis of phytate to inositol and free phosphate with release of minerals from

the phytic acid complex. An exemplary purified enzyme is a phytase derived from *Escherichia coli* B. This exemplary enzyme is shown in FIG. 1, SEQ ID NO:2.

5 The polynucleotide encoding SEQ ID NO:2 was originally recovered from genomic DNA isolated from *Escherichia coli* B as described below. It contains an open reading frame encoding a protein of 432 amino acid residues.

In one embodiment, the phytase enzyme of SEQ ID NO:2 has a molecular weight of about 47,056 kilodaltons as measured by SDS-PAGE gel
10 electrophoresis and an inferred molecular weight from the nucleotide sequence of the gene. The pI is 6.70. The pH and temperature profile and stability data for this enzyme is presented in FIG. 2. This purified enzyme may be used to catalyze the hydrolysis of phytate to inositol and free phosphate where desired. The phytase enzyme of the present invention has a high thermostability.

15

In accordance with an aspect of the present invention, there are provided isolated nucleic acid molecules (polynucleotides) which encode the mature enzyme having a deduced amino acid sequence of SEQ ID NO:2.

20

In addition, the polynucleotides of the invention (e.g., a polynucleotide having a sequence as set forth in SEQ ID NO:1) can be operably linked to a sequence encoding a second polypeptide sequence of interest to form a fusion construct. Upon expression of the fusion construct the fusion polypeptide will contain one or more moieties corresponding to a polypeptide having phytase activity
25 and the polypeptide sequence(s) of interest.

“Polynucleotide” or “nucleic acid sequence” refers to a polymeric form of nucleotides. In some instances a polynucleotide refers to a sequence that is not immediately contiguous with either of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally
30 occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a

prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. In addition, the polynucleotide sequence involved in producing a polypeptide chain can
5 include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons) depending upon the source of the polynucleotide sequence. In addition, polynucleotides greater than 100 bases long can be readily synthesized, for example, on an Applied Biosystems Model 380A DNA Synthesizer.

10 The term polynucleotide(s) generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture
15 of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions.

 In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may
20 be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

 In addition, the polynucleotides or nucleic acid sequences may contain one or
25 more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

Nucleic acid sequences can be created which encode a fusion protein and can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a coding sequence is

5 "operably linked" to another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein. An expression control sequence

10 operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic

15 acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that

20 gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

25 By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the of the polynucleotide sequence. Both

30 constitutive and inducible promoters, are included in the invention (see *e.g.*, Bitter *et al.*, Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as *pL* of bacteriophage, *plac*, *ptrp*, *ptac*

(ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

A nucleic acid sequence of the invention including, for example, a polynucleotide encoding a fusion protein, may be inserted into a recombinant expression vector. A recombinant expression vector generally refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a nucleic acid sequences. For example, a recombinant expression vector of the invention includes a polynucleotide sequence encoding an enzyme (*e.g.*, phytase) polypeptide or a fragment thereof. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al.*, *Gene* 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.* 263:3521, 1988), baculovirus-derived vectors for expression in insect cells, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV. A nucleic acid sequences of the invention can also include a localization sequence to direct the indicator to particular cellular sites by fusion to appropriate organellar targeting signals or localized host proteins. For example, a polynucleotide encoding a localization sequence, or signal sequence, can be used as a repressor and thus can be ligated or fused at the 5' terminus of a polynucleotide encoding a polypeptide of the invention such that the localization or signal peptide is located at the amino terminal end of a resulting polynucleotide/polypeptide. The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. (See, for example, Sambrook *et al.*, *Molecular Cloning --A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, and *Current Protocols in Molecular Biology*, M. Ausubel *et al.*, eds., (Current Protocols, a joint venture between Greene Publishing

Associates, Inc. and John Wiley & Sons, Inc., most recent Supplement)). These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (See also, Maniatis, *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 1989).

5 In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel, *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13, 1988; Grant, *et al.*, "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp.516-544, 1987; Glover,
10 DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3, 1986; and Bitter, "Heterologous Gene Expression in Yeast," Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684, 1987; and The Molecular Biology of the Yeast *Saccharomyces*, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II, 1982. A constitutive yeast promoter such as ADH or LEU2 or
15 an inducible promoter such as *GAL* may be used ("Cloning in Yeast," Ch. 3, R. Rothstein In: DNA Cloning Vol.11, A Practical Approach, Ed. DM Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

 An alternative expression system which could be used to express a
20 polypeptide (*e.g.*, a polypeptide encoding an enzyme) is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign or mutated polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. The sequence encoding a protein of the invention may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus
25 and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the sequences coding for a protein of the invention will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *S.*
30 *frugiperda* cells in which the inserted gene is expressed, see Smith, *et al.*, J. Virol. 46:584, 1983; Smith, U.S. Patent No. 4,215,051.

The vectors of the invention can be used to transform a host cell. By transform or transformation is meant a permanent or transient genetic change induced in a cell following incorporation of new DNA (*i.e.*, DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. A transformed cell, containing, for example, a recombinant enzyme can be incorporated into a dietary aid or delivery vehicle of the invention, for delivering the enzyme to the digestive tract of an organism.

A transformed cell or host cell generally refers to a cell (*e.g.*, prokaryote or eukaryote) into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding a polypeptide (*e.g.*, an enzyme, small molecule) or a fragment thereof.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method by procedures well known in the art. Alternatively, MgCl_2 or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, methods of transfection or transformation with DNA include calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors, as well as others known in the art, may be used. Eukaryotic cells can also be cotransfected with DNA sequences encoding an enzyme or polypeptide and a second foreign DNA molecule encoding a selectable marker, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982). The eukaryotic cell may be a yeast cell (*e.g.*, *Saccharomyces cerevisiae*), an insect cell (*e.g.*, *Drosophila sp.*) or may be a mammalian cell, including a human cell.

Eukaryotic systems, and mammalian expression systems, allow for post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells which possess the cellular machinery for processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product should be used. Post translational modification can be advantageously provided to modify the activity of a protein (*e.g.*, and enzyme) including modifying its half-life, degradation or activation. Such modifications can be advantageously performed by expressing the polypeptide or protein (*e.g.*, an enzyme) in a host cell containing the desired modification enzymes known to those of skill in the art. Such host cell lines may include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38.

Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, a polynucleotide encoding a polypeptide or protein may be ligated to an adenovirus transcription/ translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a polypeptide or fragment thereof in infected hosts (*e.g.*, see Logan & Shenk, Proc. Natl. Acad. Sci. USA, 81:3655-3659, 1984). Alternatively, the vaccinia virus 7.5K promoter may be used. (*e.g.*, see, Mackett, *et al.*, Proc. Natl. Acad. Sci. USA, 79:7415-7419, 1982; Mackett, *et al.*, J. Virol. 49:857-864, 1984; Panicali, *et al.*, Proc. Natl. Acad. Sci. USA 79:4927-4931, 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, *et al.*, Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the *neo* gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of a gene in host cells (Cone &

Mulligan, Proc. Natl. Acad. Sci. USA, 81:6349-6353, 1984). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

For long-term, high-yield production of recombinant proteins, stable
5 expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the cDNA encoding a polypeptide (*e.g.*, an enzyme) controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. The selectable marker in the recombinant vector
10 confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used,
15 including, but not limited to, the herpes simplex virus thymidine kinase (Wigler, *et al.*, Cell, 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA, 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy, *et al.*, Cell, 22:817, 1980) genes can be employed in tk-, hgp^{rt}- or ap^{rt}- cells respectively. Also, anti-metabolite resistance can be used
20 as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, *et al.*, Proc. Natl. Acad. Sci. USA, 77:3567, 1980; O'Hare, *et al.*, Proc. Natl. Acad. Sci. USA, 8:1527, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA, 78:2072, 1981; *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, J. Mol. Biol. 150:1, 1981); and
25 *hygro*, which confers resistance to hygromycin (Santerre, *et al.*, Gene 30:147, 1984) genes. Recently, additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, Proc. Natl. Acad. Sci. USA 85:8047, 1988); and *ODC* (ornithine decarboxylase) which confers resistance
30 to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, ed., 1987).

The term “primer” as used herein refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated or possible. Synthesis of a primer extension product which is complementary to a nucleic acid strand is initiated in the presence of nucleoside triphosphates and a polymerase in an appropriate buffer at a suitable temperature. For instance, if a nucleic acid sequence is inferred from a protein sequence, a primer generated to synthesize nucleic acid sequence encoding the protein sequence is actually a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One or more of the primers in this collection will be homologous with the end of the target sequence. Likewise, if a “conserved” region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences. For example, primers can be synthesized based upon the amino acid sequence of a polypeptide and can be designed based upon the degeneracy of the genetic code.

Biological molecules encoded by polynucleotides can be further mutagenized or modified to create variants conferring protease resistance, increased stability, increased or decreased activity as well as modifications to confer specialized expression and activity, for example. As described herein, a number of different mutagenesis programs, strategies and methodologies can be performed to identify and provide polypeptides (*e.g.*, enzymes) having increased stability and activity.

Variants can be produced by any number of means including methods such as, for example, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, ligation reassembly, GSSM and any combination thereof.

In one aspect, a non-stochastic method termed synthetic ligation reassembly (SLR), that is somewhat related to stochastic shuffling, save that the nucleic acid building blocks are not prepared “randomly” or stochastically (*e.g.*,

not shuffled or concatenated or chimerized randomly), but rather are prepared and assembled non-stochastically can be used to create variants for use in the invention.

5 The SLR method does not depend on the presence of a high level of homology between polynucleotides to be shuffled. The invention can be used to non-stochastically generate libraries (or sets) of progeny molecules comprised of over 10^{100} different chimeras. Conceivably, SLR can even be used to generate libraries comprised of as many as 10^{1000} different progeny chimeras or more.

10

 Thus, in one aspect, the invention provides a non-stochastic method of producing a set of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design, which method is comprised of the steps of generating by design a plurality of specific nucleic acid building blocks having
15 serviceable mutually compatible ligatable ends, and assembling these nucleic acid building blocks, such that a designed overall assembly order is achieved.

 The mutually compatible ligatable ends of the nucleic acid building blocks to be assembled are considered to be “serviceable” for this type of ordered
20 assembly if they enable the building blocks to be coupled in predetermined orders. Thus, in one aspect, the overall assembly order in which the nucleic acid building blocks can be coupled is specified by the design of the ligatable ends and, if more than one assembly step is to be used, then the overall assembly order in which the nucleic acid building blocks can be coupled is also specified by the sequential
25 order of the assembly step(s). In a one embodiment of the invention, the annealed building pieces are treated with an enzyme, such as a ligase (*e.g.*, T4 DNA ligase) to achieve covalent bonding of the building pieces.

 In a another embodiment, the design of nucleic acid building blocks is
30 obtained upon analysis of the sequences of a set of progenitor nucleic acid templates that serve as a basis for producing a progeny set of finalized chimeric nucleic acid molecules. These progenitor nucleic acid templates thus serve as a

source of sequence information that aids in the design of the nucleic acid building blocks that are to be mutagenized, *i.e.* chimerized or shuffled.

In one exemplification, the invention provides for the chimerization of a family of related genes and their encoded family of related products. In a particular exemplification, the encoded products are enzymes. Enzymes and polypeptides for use in the invention can be mutagenized in accordance with the methods described herein.

Thus according to one aspect of the invention, the sequences of a plurality of progenitor nucleic acid templates are aligned in order to select one or more demarcation points, which demarcation points can be located at an area of homology. The demarcation points can be used to delineate the boundaries of nucleic acid building blocks to be generated. Thus, the demarcation points identified and selected in the progenitor molecules serve as potential chimerization points in the assembly of the progeny molecules.

If desired a demarcation point can be comprised of no (*i.e.*, zero) homologous nucleotides. In this case, ligation can be blunt, or sticky ends can be introduced (*e.g.*, degenerate codons that don't alter the amino acid's coded thereby) or addition of nucleotide that serve as linkers.

Typically a serviceable demarcation point is an area of homology (comprised of at least one homologous nucleotide base) shared by at least two progenitor templates, but the demarcation point can be an area of homology that is shared by at least half of the progenitor templates, at least two thirds of the progenitor templates, at least three fourths of the progenitor templates, and preferably at almost all of the progenitor templates. Even more preferably still a serviceable demarcation point is an area of homology that is shared by all of the progenitor templates.

In a one embodiment, the ligation reassembly process is performed exhaustively in order to generate an exhaustive library. In other words, all possible ordered combinations of the nucleic acid building blocks are represented in the set of finalized chimeric nucleic acid molecules. At the same time, the assembly order (*i.e.* the order of assembly of each building block in the 5' to 3' sequence of each finalized chimeric nucleic acid) in each combination is by design (or non-stochastic). Because of the non-stochastic nature of the method, the possibility of unwanted side products is greatly reduced.

10 In another embodiment, the method provides that, the ligation reassembly process is performed systematically, for example in order to generate a systematically compartmentalized library, with compartments that can be screened systematically, *e.g.*, one by one. In other words the invention provides that, through the selective and judicious use of specific nucleic acid building blocks, 15 coupled with the selective and judicious use of sequentially stepped assembly reactions, an experimental design can be achieved where specific sets of progeny products are made in each of several reaction vessels. This allows a systematic examination and screening procedure to be performed. Thus, it allows a potentially very large number of progeny molecules to be examined systematically 20 in smaller groups.

Because of its ability to perform chimerizations in a manner that is highly flexible yet exhaustive and systematic as well, particularly when there is a low level of homology among the progenitor molecules, the instant invention provides 25 for the generation of a library (or set) comprised of a large number of progeny molecules. Because of the non-stochastic nature of the instant ligation reassembly invention, the progeny molecules generated preferably comprise a library of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design. In a particularly embodiment, such a generated library is 30 comprised of greater than 10^3 to greater than 10^{1000} different progeny molecular species.

In one aspect, a set of finalized chimeric nucleic acid molecules, produced as described is comprised of a polynucleotide encoding a polypeptide. According to one embodiment, this polynucleotide is a gene, which may be a man-made gene. According to another embodiment, this polynucleotide is a gene pathway, which may be a man-made gene pathway. The invention provides that one or more man-made genes generated by the invention may be incorporated into a man-made gene pathway, such as pathway operable in a eukaryotic organism (including a plant).

In another exemplification, the synthetic nature of the step in which the building blocks are generated allows the design and introduction of nucleotides (*e.g.*, one or more nucleotides, which may be, for example, codons or introns or regulatory sequences) that can later be optionally removed in an *in vitro* process (*e.g.*, by mutagenesis) or in an *in vivo* process (*e.g.*, by utilizing the gene splicing ability of a host organism). It is appreciated that in many instances the introduction of these nucleotides may also be desirable for many other reasons in addition to the potential benefit of creating a serviceable demarcation point.

Thus, according to another embodiment, the invention provides that a nucleic acid building block can be used to introduce an intron. Thus, the invention provides that functional introns may be introduced into a man-made gene of the invention. The invention also provides that functional introns may be introduced into a man-made gene pathway of the invention. Accordingly, the invention provides for the generation of a chimeric polynucleotide that is a man-made gene containing one (or more) artificially introduced intron(s).

Accordingly, the invention also provides for the generation of a chimeric polynucleotide that is a man-made gene pathway containing one (or more) artificially introduced intron(s). Preferably, the artificially introduced intron(s) are functional in one or more host cells for gene splicing much in the way that naturally-occurring introns serve functionally in gene splicing. The invention

provides a process of producing man-made intron-containing polynucleotides to be introduced into host organisms for recombination and/or splicing.

A man-made genes produced using the invention can also serve as a
5 substrate for recombination with another nucleic acid. Likewise, a man-made
gene pathway produced using the invention can also serve as a substrate for
recombination with another nucleic acid. In a preferred instance, the
recombination is facilitated by, or occurs at, areas of homology between the man-
made intron-containing gene and a nucleic acid which serves as a recombination
10 partner. In a particularly preferred instance, the recombination partner may also
be a nucleic acid generated by the invention, including a man-made gene or a
man-made gene pathway. Recombination may be facilitated by or may occur at
areas of homology that exist at the one (or more) artificially introduced intron(s)
in the man-made gene.

15

The synthetic ligation reassembly method of the invention utilizes a
plurality of nucleic acid building blocks, each of which preferably has two
ligatable ends. The two ligatable ends on each nucleic acid building block may be
two blunt ends (*i.e.* each having an overhang of zero nucleotides), or preferably
20 one blunt end and one overhang, or more preferably still two overhangs.

A serviceable overhang for this purpose may be a 3' overhang or a 5'
overhang. Thus, a nucleic acid building block may have a 3' overhang or
alternatively a 5' overhang or alternatively two 3' overhangs or alternatively two
25 5' overhangs. The overall order in which the nucleic acid building blocks are
assembled to form a finalized chimeric nucleic acid molecule is determined by
purposeful experimental design and is not random.

According to one preferred embodiment, a nucleic acid building block is
30 generated by chemical synthesis of two single-stranded nucleic acids (also
referred to as single-stranded oligos) and contacting them so as to allow them to
anneal to form a double-stranded nucleic acid building block.

A double-stranded nucleic acid building block can be of variable size. The sizes of these building blocks can be small or large. Preferred sizes for building block range from 1 base pair (not including any overhangs) to 100,000 base pairs
5 (not including any overhangs). Other preferred size ranges are also provided, which have lower limits of from 1 bp to 10,000 bp (including every integer value in between), and upper limits of from 2 bp to 100, 000 bp (including every integer value in between).

10 Many methods exist by which a double-stranded nucleic acid building block can be generated that is serviceable for the invention; and these are known in the art and can be readily performed by the skilled artisan.

According to one embodiment, a double-stranded nucleic acid building
15 block is generated by first generating two single stranded nucleic acids and allowing them to anneal to form a double-stranded nucleic acid building block. The two strands of a double-stranded nucleic acid building block may be complementary at every nucleotide apart from any that form an overhang; thus containing no mismatches, apart from any overhang(s). According to another
20 embodiment, the two strands of a double-stranded nucleic acid building block are complementary at fewer than every nucleotide apart from any that form an overhang. Thus, according to this embodiment, a double-stranded nucleic acid building block can be used to introduce codon degeneracy. Preferably the codon degeneracy is introduced using the site-saturation mutagenesis described herein,
25 using one or more N,N,G/T cassettes or alternatively using one or more N,N,N cassettes.

The *in vivo* recombination method of the invention can be performed blindly on a pool of unknown hybrids or alleles of a specific polynucleotide or
30 sequence. However, it is not necessary to know the actual DNA or RNA sequence of the specific polynucleotide.

The approach of using recombination within a mixed population of genes can be useful for the generation of any useful proteins, for example, interleukin I, antibodies, tPA and growth hormone. This approach may be used to generate proteins having altered specificity or activity. The approach may also be useful
5 for the generation of hybrid nucleic acid sequences, for example, promoter regions, introns, exons, enhancer sequences, 3' untranslated regions or 5' untranslated regions of genes. Thus this approach may be used to generate genes having increased rates of expression. This approach may also be useful in the study of repetitive DNA sequences. Finally, this approach may be useful to
10 mutate ribozymes or aptamers.

In one aspect the invention described herein is directed to the use of repeated cycles of reductive reassortment, recombination and selection which allow for the directed molecular evolution of highly complex linear sequences,
15 such as DNA, RNA or proteins through recombination.

In vivo shuffling of molecules is useful in providing variants and can be performed utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity,
20 genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the chiasma requires the recognition of homologous sequences.

25
There are a number of methods known to one of skill in the art for producing enzymes for use with the compositions of the invention. For example, superior or modified enzymes can be produced using hybrid polynucleotides which may encode biologically active hybrid polypeptides (e.g., hybrid phytases). In
30 one aspect, the original polynucleotides encode biologically active polypeptides. New hybrid polypeptides can be produced by utilizing cellular processes which integrate the sequence of the original polynucleotides such that the resulting

hybrid polynucleotide encodes a polypeptide demonstrating activities derived from the original biologically active polypeptides. For example, the original polynucleotides may encode a particular enzyme from different microorganisms. An enzyme encoded by a first polynucleotide from one organism or variant may, 5 for example, function effectively under a particular environmental condition, *e.g.* low pH. An enzyme encoded by a second polynucleotide from a different organism or variant may function effectively under a different environmental condition, such as extremely high temperatures. A hybrid polynucleotide containing sequences from the first and second original polynucleotides may 10 encode an enzyme which exhibits characteristics of both enzymes encoded by the original polynucleotides. Thus, the enzyme encoded by the hybrid polynucleotide may function effectively under environmental conditions shared by each of the enzymes encoded by the first and second polynucleotides, *e.g.*, low pH and extreme temperatures.

15

Enzymes applicable to the invention and the polynucleotides encoding them include, enzymes belonging to the following enzymes classes: E.C.1. Oxidoreductases; E.C.2. Transferases; E.C.3. Hydrolases.; E.C.4. Lyases; E.C.5. Isomerases; and E.C.6. Ligases. More specifically the enzymes include, but are 20 not limited to, phytases, amylases, hydrolases, cellulases, and glucosidases. A hybrid polypeptide resulting from the method of the invention may exhibit specialized enzyme activity not displayed in the original enzymes. For example, following recombination and/or reductive reassortment of polynucleotides encoding phytase activities, the resulting hybrid polypeptide encoded by a hybrid 25 polynucleotide can be screened for specialized phytase activities obtained from each of the original enzymes, *i.e.* the type of molecule on which the phytase acts and the temperature at which the phytase functions. Thus, for example, the phytase may be screened to ascertain those chemical functionalities which distinguish the hybrid phytase from the original phytases, such as: (a) amide 30 (peptide bonds), *i.e.*, susceptibility to proteases; and (b) the temperature, pH or salt concentration at which the hybrid polypeptide functions. The invention also envisions renaturable enzymes (*e.g.*, enzymes that denature and renature at

defined pH or temperature ranges) that become active only at the proper pH or temperature.

Sources of the original polynucleotides may be isolated from individual
5 organisms ("isolates"), collections of organisms that have been grown in defined
media ("enrichment cultures"), known sequence available in databases available
to those of skill in the art, or, uncultivated organisms ("environmental samples").
The use of a culture-independent approach to derive polynucleotides encoding
novel bioactivities from environmental samples is most preferable since it allows
10 one to access untapped resources of biodiversity.

"Environmental libraries" are generated from environmental samples and
represent the collective genomes of naturally occurring organisms archived in
cloning vectors that can be propagated in suitable prokaryotic hosts. Because the
15 cloned DNA is initially extracted directly from environmental samples, the
libraries are not limited to the small fraction of prokaryotes that can be grown in
pure culture. Additionally, a normalization of the environmental DNA present in
these samples could allow more equal representation of the DNA from all of the
species present in the original sample. This can dramatically increase the
20 efficiency of finding interesting genes from minor constituents of the sample
which may be under-represented by several orders of magnitude compared to the
dominant species.

For example, gene libraries generated from one or more uncultivated
25 microorganisms are screened for an activity of interest. Potential pathways
encoding bioactive molecules of interest are first captured in prokaryotic cells in
the form of gene expression libraries. Polynucleotides encoding activities of
interest are isolated from such libraries and introduced into a host cell. The host
cell is grown under conditions which promote recombination and/or reductive
30 reassortment creating potentially active biomolecules with novel or enhanced
activities.

The microorganisms from which the polynucleotide may be prepared include prokaryotic microorganisms, such as *Eubacteria* and *Archaeobacteria*, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Polynucleotides may be isolated from environmental samples in which case the nucleic acid may be recovered without culturing of an organism or recovered from one or more cultured organisms. In one aspect, such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles and acidophiles. Polynucleotides encoding enzymes isolated from extremophilic microorganisms are particularly useful. Such enzymes may function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead Sea, at pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH values greater than 11 in sewage sludge. For example, several esterases and lipases cloned and expressed from extremophilic organisms show high activity throughout a wide range of temperatures and pHs.

In another aspect, it is envisioned that novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof can be generated for use in a composition of the invention. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are

multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating
5 genetic manipulation and *in vitro* studies of these genes/proteins.

Gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-
10 related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affects high-frequency transfer of itself during conjugation and
15 is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. A particularly preferred embodiment is to use cloning vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. coli* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a
20 mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library." Another type of vector for use in the present invention is a cosmid vector. Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. Cloning into cosmid vectors is described in detail in "Molecular Cloning: A
25 laboratory Manual" (Sambrook *et al.*, 1989). Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster
30 can then be screened for enhanced activities not found in the original gene clusters.

Therefore, methods for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity or stability can be performed to identify and incorporate biologically active polypeptides into the compositions of the invention. Such methods include:

- 5 1) introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;
- 10 2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;
- 3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;
- 4) screening the hybrid polypeptide under conditions which promote
15 identification of enhanced biological activity; and
- 5) isolating the a polynucleotide encoding the hybrid polypeptide.

Methods for screening for various enzyme activities are known to those of skill in the art and are discussed throughout the present specification. Such
20 methods may be employed when isolating the polypeptides and polynucleotides for use with the compositions of the invention.

As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids,
25 cosmids, fosmids, bacterial artificial chromosomes, viral DNA (e.g., vaccinia, adenovirus, fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as *bacillus*, *aspergillus* and yeast). Thus, for example, the DNA may be included in any one of a variety
30 of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially

available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia).

- 5 However, any other plasmid or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the invention.

The DNA sequence in the expression vector is operatively linked to an
10 appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include *lacI*, *lacZ*, *T3*, *T7*, *gpt*, *lambda P_R*, *P_L* and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of
15 ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. In addition, the expression
20 vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

25 *In vivo* reassortment is focused on “inter-molecular” processes collectively referred to as “recombination” which in bacteria, is generally viewed as a “RecA-dependent” phenomenon. The invention can rely on recombination processes of a host cell to recombine and re-assort sequences, or the cells’ ability to mediate reductive processes to decrease the complexity of quasi-repeated sequences in the
30 cell by deletion. This process of “reductive reassortment” occurs by an “intra-molecular”, RecA-independent process.

Therefore, in another aspect of the invention, novel polynucleotides can be generated by the process of reductive reassortment and used in the compositions or to produce polypeptides used in the compositions of the invention. The method involves the generation of constructs containing consecutive sequences (original
5 encoding sequences), their insertion into an appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or
10 reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may
15 involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

Repeated or "quasi-repeated" sequences play a role in genetic instability. "Quasi-repeats" are repeats that are not restricted to their original unit structure.
20 Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive sequences become essentially invisible and the quasi-repetitive nature of the resulting construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct
25 operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion) events can occur virtually anywhere within the quasi-repetitive units.

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When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units.

Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the inversion delineates the endpoints of the adjacent unit so that deletion formation will favor the loss of discrete units. Thus, it is preferable with the present method that the sequences are in the same orientation. Random orientation of quasi-repeated sequences will result in the loss of reassortment efficiency, while consistent orientation of the sequences will offer the highest efficiency. However, while having fewer of the contiguous sequences in the same orientation decreases the efficiency, it may still provide sufficient elasticity for the effective recovery of novel molecules. Constructs can be made with the quasi-repeated sequences in the same orientation to allow higher efficiency.

Sequences can be assembled in a head to tail orientation using any of a variety of methods, including the following:

- a) Primers that include a poly-A head and poly-T tail which when made single-stranded would provide orientation can be utilized. This is accomplished by having the first few bases of the primers made from RNA and hence easily removed RNaseH.
- b) Primers that include unique restriction cleavage sites can be utilized. Multiple sites, a battery of unique sequences, and repeated synthesis and ligation steps would be required.
- c) The inner few bases of the primer could be thiolated and an exonuclease used to produce properly tailed molecules.

The recovery of the re-assorted sequences relies on the identification of cloning vectors with a reduced RI. The re-assorted encoding sequences can then be recovered by amplification. The products are re-cloned and expressed. The recovery of cloning vectors with reduced RI can be effected by:

- 1) The use of vectors only stably maintained when the construct is reduced in complexity.
- 2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid

isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.

- 3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.
- 5 4) The use of direct selection techniques with an expression vector and the appropriate selection.

Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products.
10 These types of sequences are particularly useful in the invention as quasi-repeats. The process is not limited to nearly identical repeats.

The following example demonstrates a method of producing variants for use in the invention. Encoding nucleic acid sequences (quasi-repeats) derived
15 from three (3) unique species are used. Each sequence encodes a protein with a distinct set of properties. Each of the sequences differs by a single or a few base pairs at a unique position in the sequence which are designated "A", "B" and "C". The quasi-repeated sequences are separately or collectively amplified and ligated into random assemblies such that all possible permutations and combinations are
20 available in the population of ligated molecules. The number of quasi-repeat units can be controlled by the assembly conditions. The average number of quasi-repeated units in a construct is defined as the repetitive index (RI).

Once formed, the constructs may, or may not be size fractionated on an
25 agarose gel according to published protocols, inserted into a cloning vector, and transfected into an appropriate host cell. The cells are then propagated and "reductive reassortment" is effected. The rate of the reductive reassortment process may be stimulated by the introduction of DNA damage if desired. Whether the reduction in RI is mediated by deletion formation between repeated
30 sequences by an "intra-molecular" mechanism, or mediated by recombination-like events through "inter-molecular" mechanisms is immaterial. The end result is a reassortment of the molecules into all possible combinations.

Optionally, the method comprises the additional step of screening the library members of the shuffled pool to identify individual shuffled library members having the ability to bind or otherwise interact, or catalyze a particular reaction (e.g., such as catalytic domain of an enzyme) with a predetermined macromolecule, such as for example a proteinaceous molecule, an oligosaccharide, viron, or other predetermined compound or structure.

The polypeptides that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes (e.g., as a feed additive, catalysts, solutes for increasing osmolarity of an aqueous solution, and the like), and/or can be subjected to one or more additional cycles of shuffling and/or selection.

It is envisioned that prior to or during recombination or reassortment, polynucleotides generated by the method of the invention can be subjected to agents or processes which promote the introduction of mutations into the original polynucleotides. The introduction of such mutations would increase the diversity of resulting hybrid polynucleotides and polypeptides encoded therefrom. The agents or processes which promote mutagenesis can include, but are not limited to: (+)-CC-1065, or a synthetic analog such as (+)-CC-1065-(N3-Adenine, see Sun and Hurley, 1992); an N-acetylated or deacetylated 4'-fluoro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see, for example, van de Poll *et al.*, 1992); or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see also, van de Poll *et al.*, 1992, pp. 751-758); trivalent chromium, a trivalent chromium salt, a polycyclic aromatic hydrocarbon ("PAH") DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[*a*]anthracene ("BMA"), tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"), 2-bromoacrolein (2BA), benzo[*a*]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"), a platinum(II) halogen salt, N-hydroxy-2-amino-3-methylimidazo[4,5-*f*]-quinoline ("N-hydroxy-IQ"), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*f*]-pyridine ("N-hydroxy-

PhIP”). Especially preferred means for slowing or halting PCR amplification consist of UV light (+)-CC-1065 and (+)-CC-1065-(N3-Adenine). Particularly encompassed means are DNA adducts or polynucleotides comprising the DNA adducts from the polynucleotides or polynucleotide pool, which can be released or
5 removed by a process including heating the solution containing the polynucleotides prior to further processing.

The invention also utilizes recombinant proteins developed by treating a sample containing double-stranded template polynucleotides encoding a wild-type
10 protein under conditions which provide for the production of hybrid or re-assorted polynucleotides.

Polypeptides and polynucleotides used in the invention can also be provided by the use of proprietary codon primers (containing a degenerate N,N,N
15 sequence) to introduce point mutations into a polynucleotide, so as to generate a set of progeny polypeptides in which a full range of single amino acid substitutions is represented at each amino acid position (gene site saturated mutagenesis (GSSM)). The oligos used are comprised contiguously of a first homologous sequence, a degenerate N,N,N sequence, and preferably but not
20 necessarily a second homologous sequence. The downstream progeny translational products from the use of such oligos include all possible amino acid changes at each amino acid site along the polypeptide, because the degeneracy of the N,N,N sequence includes codons for all 20 amino acids.

25 In one aspect, one such degenerate oligo (comprised of one degenerate N,N,N cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate N,N,N cassettes are used – either in the same oligo or not, for subjecting at least two original codons in a parental polynucleotide template to
30 a full range of codon substitutions. Thus, more than one N,N,N sequence can be contained in one oligo to introduce amino acid mutations at more than one site. This plurality of N,N,N sequences can be directly contiguous, or separated by one

or more additional nucleotide sequence(s). In another aspect, oligos serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,N sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

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In a particular exemplification, it is possible to simultaneously mutagenize two or more contiguous amino acid positions using an oligo that contains contiguous N,N,N triplets, *i.e.* a degenerate (N,N,G/T)_n sequence.

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Degenerate cassettes having less degeneracy than the N,N,G/T sequence can also be used to create variants. For example, it may be desirable in some instances to use (*e.g.* in an oligo) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other bases including any combinations and permutations thereof can be used in the remaining two positions of the triplet. Alternatively, it may be desirable in some instances to use (*e.g.*, in an oligo) a degenerate N,N,N triplet sequence, or an N,N, G/C triplet sequence.

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It is appreciated, however, that the use of a degenerate triplet (such as N,N,G/T or an N,N, G/C triplet sequence) as disclosed herein is advantageous for several reasons. In one aspect, the degenerate triplet provides a means to systematically and fairly easily generate the substitution of the full range of possible amino acids (for a total of 20 amino acids) into each and every amino acid position in a polypeptide. Thus, for a 100 amino acid polypeptide, the invention provides a way to systematically and fairly easily generate 2000 distinct species (*i.e.*, 20 possible amino acids per position times 100 amino acid positions). It is appreciated that there is provided, through the use of an oligo containing a degenerate N,N,G/T or an N,N, G/C triplet sequence, 32 individual sequences that code for 20 possible amino acids. Thus, in a reaction vessel in which a parental polynucleotide sequence is subjected to saturation mutagenesis using one such oligo, there are generated 32 distinct progeny polynucleotides encoding 20 distinct polypeptides. In contrast, the use of a non-degenerate oligo in site-directed

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mutagenesis leads to only one progeny polypeptide product per reaction vessel. When an N,N,N sequence is utilized, 64 individual sequences are provided.

Nondegenerate oligos, which can optionally be used in combination with
5 degenerate primers can also be used to develop polypeptides and polynucleotides for use in the invention. It is appreciated that in some situations, it is advantageous to use nondegenerate oligos to generate specific point mutations in a working polynucleotide. This provides a means to generate specific silent point mutations, point mutations leading to corresponding amino acid changes, and point mutations
10 that cause the generation of stop codons and the corresponding expression of polypeptide fragments. Such techniques are useful in eliminating protease recognition sequences, for example, in order to increase the stability or half-life of a polypeptide (*e.g.*, an enzyme).

15 Thus, each saturation mutagenesis reaction vessel contains polynucleotides encoding at least 20 progeny polypeptide molecules such that all 20 amino acids are represented at the one specific amino acid position corresponding to the codon position mutagenized in the parental polynucleotide. The 32-fold degenerate progeny polypeptides generated from each saturation mutagenesis reaction vessel
20 can be subjected to clonal amplification (*e.g.*, cloned into a suitable *E. coli* host using an expression vector) and subjected to expression screening. When an individual progeny polypeptide is identified by screening to display a favorable change in property (when compared to the parental polypeptide), it can be sequenced to identify the correspondingly favorable amino acid substitution
25 contained therein.

It is appreciated that upon mutagenizing each and every amino acid position in a parental polypeptide using saturation mutagenesis as disclosed herein, favorable amino acid changes may be identified at more than one amino
30 acid position. One or more new progeny molecules can be generated that contain a combination of all or part of these favorable amino acid substitutions. For example, if 2 specific favorable amino acid changes are identified in each of 3

amino acid positions in a polypeptide, the permutations include 3 possibilities at each position (no change from the original amino acid, and each of two favorable changes) and 3 positions. Thus, there are 3 x 3 x 3 or 27 total possibilities, including 7 that were previously examined - 6 single point mutations (*i.e.*, 2 at
5 each of three positions) and no change at any position.

In addition, site-saturation mutagenesis can be used together with shuffling, chimerization, recombination and other mutagenizing processes, along with screening. The invention provides for the use of any mutagenizing
10 process(es), including saturation mutagenesis, in an iterative manner to create polypeptides (*e.g.*, enzymes) and polynucleotides for use in the invention. In one exemplification, the iterative use of any mutagenizing process(es) is used in combination with screening.

15 Thus, the invention provides for the use of saturation mutagenesis in combination with additional mutagenization processes, such as a process where two or more related polynucleotides are introduced into a suitable host cell such that a hybrid polynucleotide is generated by recombination and reductive
reassortment.

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In addition to performing mutagenesis along the entire sequence of a gene, mutagenesis can be used to replace each of any number of bases in a polynucleotide sequence, wherein the number of bases to be mutagenized is preferably every integer from 15 to 100,000. Thus, instead of mutagenizing every
25 position along a molecule, one can subject every or a discrete number of bases (preferably a subset totaling from 15 to 100,000) to mutagenesis. Preferably, a separate nucleotide is used for mutagenizing each position or group of positions along a polynucleotide sequence. A group of 3 positions to be mutagenized may be a codon. The mutations are preferably introduced using a mutagenic primer,
30 containing a heterologous cassette, also referred to as a mutagenic cassette. Typical cassettes can have from 1 to 500 bases. Each nucleotide position in such heterologous cassettes can be N, A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T,

C/G/T, A/G/T, A/C/T, A/C/G, or E, where E is any base that is not A, C, G, or T (E can be referred to as a designer oligo).

In a general sense, saturation mutagenesis is comprised of mutagenizing a
5 complete set of mutagenic cassettes (wherein each cassette is preferably about 1-
500 bases in length) in defined polynucleotide sequence to be mutagenized
(wherein the sequence to be mutagenized is preferably from about 15 to 100,000
bases in length). Thus, a group of mutations (ranging from 1 to 100 mutations) is
introduced into each cassette to be mutagenized. A grouping of mutations to be
10 introduced into one cassette can be different or the same from a second grouping
of mutations to be introduced into a second cassette during the application of one
round of saturation mutagenesis. Such groupings are exemplified by deletions,
additions, groupings of particular codons, and groupings of particular nucleotide
cassettes.

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Defined sequences to be mutagenized include a whole gene, pathway,
cDNA, an entire open reading frame (ORF), an entire promoter, enhancer,
repressor/transactivator, origin of replication, intron, operator, or any
polynucleotide functional group. Generally, a "defined sequences" for this
20 purpose may be any polynucleotide, for example, a 15 base-polynucleotide
sequence, and polynucleotide sequences of lengths between 15 bases and 15,000
bases (the invention specifically names every integer in between). Considerations
in choosing groupings of codons include types of amino acids encoded by a
degenerate mutagenic cassette.

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In a one exemplification a grouping of mutations that can be introduced
into a mutagenic cassette provides for degenerate codon substitutions (using
degenerate oligos) that code for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,
18, 19, and 20 amino acids at each position, and a library of polypeptides encoded
30 thereby.

Polypeptides

The invention also provides polypeptides (*e.g.*, enzymes, and more particular a phytase) for use in the compositions of the invention. The enzymes are modified through any of a number of methodologies (as described herein) to increase their activity, stability, protease resistance or other functional characteristics. For example, a phytase enzyme has a sequence as set forth in SEQ ID NO:2. The polypeptides or enzymes may also be operably linked to a polypeptide of interest to form a fusion protein as described herein. In addition, a polypeptide of the invention can include a peptide or polypeptide sequence that targets the polypeptide sequence to a particular organelle, subcellular compartment, tissue, or cell type. Such modifications are within the scope of the invention and are based upon the ability to link amino acid sequences to the N-terminal or C-terminal region of the polypeptide.

A “polypeptide” or “protein” refers to a polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being typical. D-optical isomers may be preferred under some conditions as they increase their stability (*e.g.*, by increasing protease resistance). A polypeptide, as used herein, is intended to encompass any amino acid sequence and include modified sequences such as glycoproteins, which provides a polypeptide having the desired biological activity or an activity associated with the polypeptide from which it is derived. Accordingly, the polypeptides for use in the compositions of the invention are intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically synthesized. In one embodiment, the polypeptide is an enzyme having phytase activity. Polypeptide or protein fragments are also encompassed by the invention. Fragments can have the same or substantially the same amino acid sequence as the naturally occurring protein. A polypeptide or peptide having substantially the same sequence means that an amino acid sequence is largely, but not entirely, the same, but retains a functional activity of the sequence to which it is related. In general, two amino acid sequences

are substantially the same or substantially homologous if they are at least 70% identical.

Homology or identity is often measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms “homology” and “identity” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol 48:443, 1970, by the search for similarity method of Person & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

On example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402, 1977, and Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990, respectively. Software for performing BLAST analyses is publicly available through the National Center for
5 Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*,
10 *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid
15 sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm
20 parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff &
25 Henikoff, Proc. Natl. Acad. Sci. USA 89:10915, 1989, alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873, 1993). One measure of similarity provided by BLAST algorithm is the
30 smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if

the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

A polypeptide may be substantially related but for a conservative variation, such polypeptides being encompassed by the invention. A conservative variation denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Other illustrative examples of conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Modifications and substitutions are not limited to replacement of amino acids. For a variety of purposes, such as increased stability, solubility, or configuration concerns, one skilled in the art will recognize the need to introduce, (by deletion, replacement, or addition) other modifications. Examples of such other modifications include incorporation of rare amino acids, dextra-amino acids, glycosylation sites, cytosine for specific disulfide bridge formation. The modified peptides can be chemically synthesized, or the isolated gene can be site-directed mutagenized, or a synthetic gene can be synthesized and expressed in bacteria, yeast, baculovirus, tissue culture and so on. Whether a change results in a functioning peptide can readily be determined by direct analysis for function in an assay that

relies on ability of the modified enzyme (or fragment) to carry out the normal function of the natural phytase enzyme (or fragment). For example, modified peptides can be tested for their ability to catalyze an enzymatic reaction. Such assays measure the production of an enzymatic product or the decrease in the
5 presence of a substrate.

Generally, polypeptides have a half-life once administered, and are degraded by endogenous proteases. The therapeutic potential of such polypeptides would be dramatically increased by extension of the *in vivo* half-life. In addition to increasing the half-life of peptides, other characteristics of peptides might be
10 changed in ways which would provide useful pharmaceutically active compounds, *e.g.*, improving binding affinity. Some of the general means contemplated for the modification of peptides are outlined in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins--A survey of Recent Developments, Weinstein, B., ed., Marcel Dekker, Inc., publ., New York (1983), which is incorporated herein by
15 reference to disclose methods of modifying amino acids and peptides.

The substitution of D-amino acids for the normal L-stereoisomer has been shown to increase half-life. The theory of this approach is that the proteolytic enzymes responsible for cleavage may be stereospecific, so that substituting a D-amino acid may render the peptide unacceptable as a cleavage substrate.

20 In addition, the stability or resistance of polypeptides to protease can be altered, for example, by replacing the amide with a saturated amine. See, for example, Skiles *et al.*, U.S. Pat. No. 4,496,542. J. S. Kaltenbronn *et al.*, "Proceedings of the 11th American Peptide Symposium" (ESCOM Science Publishers, The Netherlands, 1990) pp. 969-70, which discloses peptides in which
25 all of the peptide bonds were replaced with saturated amine bonds.

In a further embodiment, a polypeptide for use in the compositions of the invention can be derivatized by acetylation or alkylation. J. T. Suh *et al.*, Eur J Med Chem--Chim Ther 20:563-70, 1985, which discloses Lys-Gly dipeptide derivatives for inhibition of angiotensin-converting enzyme, in which the Gly
30 amide nitrogen was substituted with 2,3-dihydro-1H-indene. Sempuku *et al.*, JP

58/150,562 (Chem Abs (1984) 100: 68019b), discloses N-substituted glycine derivatives useful for inhibition of angiotensin-converting enzyme. J. D. Young *et al.*, "Proceedings of the 11th American Peptide Symposium" (ESCOM Science Publishers, The Netherlands, 1990) pp. 155-56, discloses a synthesis of
5 bradykinin, in which proline at position 7 was replaced by N-benzylglycine.

Solid-phase chemical peptide synthesis methods can also be used to synthesize the polypeptide or fragments of the invention. Such method have been known in the art since the early 1960's (Merrifield, R. B., *J. Am. Chem. Soc.*, 85, 2149-2154 (1963) (See also Stewart, J. M. and Young, J. D., *Solid Phase Peptide*
10 *Synthesis*, 2 ed., Pierce Chemical Co., Rockford, Ill., pp. 11-12)) and have recently been employed in commercially available laboratory peptide design and synthesis kits (Cambridge Research Biochemicals). Such commercially available laboratory kits have generally utilized the teachings of H. M. Geysen *et al*, *Proc. Natl. Acad. Sci., USA*, 81, 3998 (1984) and provide for synthesizing peptides upon the tips of a
15 multitude of "rods" or "pins" all of which are connected to a single plate. When such a system is utilized, a plate of rods or pins is inverted and inserted into a second plate of corresponding wells or reservoirs, which contain solutions for attaching or anchoring an appropriate amino acid to the pin's or rod's tips. By repeating such a process step, *i.e.*, inverting and inserting the rod's and pin's tips into appropriate
20 solutions, amino acids are built into desired peptides. In addition, a number of available Fmoc peptide synthesis systems are available. For example, assembly of a polypeptide or fragment can be carried out on a solid support using an Applied Biosystems, Inc. Model 431A automated peptide synthesizer. Such equipment provides ready access to the peptides of the invention, either by direct synthesis or
25 by synthesis of a series of fragments that can be coupled using other known techniques.

Nomenclature and Position Specificity of Phytases

In the present context a phytase is an enzyme which catalyzes the hydrolysis of phytate (myo-inositol hexakisphosphate) to (1) myo-inositol; (2)
30 mono-, di-, tri-, tetra- and/or penta-phosphates thereof; and/or (3) inorganic phosphate. The above compounds are sometimes referred to herein as IP6, I, IP1,

IP2, IP3, IP4, IP5 and P, respectively. This means that by action of a phytase, IP6 is degraded into P+one or more of the components IP5, IP4, IP3, IP2, IP1 and I.

Alternatively, myo-inositol carrying in total n phosphate groups attached to positions $p, q, r, \dots$ is denoted $\text{Ins}(p,q,r, \dots)\text{P}_n$. For convenience

5 $\text{Ins}(1,2,3,4,5,6)\text{P}_6$ (phytic acid) is abbreviated PA.

According to the Enzyme nomenclature database ExPASy (a repository of information relative to the nomenclature of enzymes primarily based on the recommendations of the Nomenclature Committee of the International Union of
10 Biochemistry and Molecular Biology (IUBMB) describing each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided), a number of different types of phytases are known, for example, a 3-phytase (myo-inositol hexaphosphate 3-phosphohydrolase, EC 3.1.3.8) and a 6-phytase (myo-inositol hexaphosphate 6-phosphohydrolase, EC 3.1.3.26). The 3-
15 phytase hydrolyses first the ester bond at the 3-position, whereas the 6-phytase hydrolyzes first the ester bond at the 6-position.

Inositol phosphate Nomenclature

Considering the primary hydrolysis products of a phytase acting on phytic
20 acid, some of the resulting esters are diastereomers and some are enantiomers. Generally, it is easier to discriminate between diastereomers, since they have different physical properties, whereas it is more difficult to discriminate between enantiomers which are mirror images of each other.

25 Thus, $\text{Ins}(1,2,4,5,6)\text{P}_5$ (3-phosphate removed) and $\text{Ins}(1,2,3,4,5)\text{P}_5$ (6-phosphate removed) are diastereomers and easy to discriminate, whereas $\text{Ins}(1,2,4,5,6)\text{P}_5$ (3-phosphate removed) and $\text{Ins}(2,3,4,5,6)\text{P}_5$ (1-phosphate removed) are enantiomers. The same holds true for the pair $\text{Ins}(1,2,3,4,5)\text{P}_5$ (6-phosphate removed) and $\text{Ins}(1,2,3,5,6)\text{P}_5$ (4-phosphate removed). Accordingly, of
30 the 6 penta-phosphate esters resulting from the first step of the phytase catalyzed hydrolysis of phytic acid, you can only discriminate easily between those esters in which the 2-, 3-, 5- and 6-phosphate has been removed, *i.e.*, you have four

diastereomers only, each of the remaining two esters being an enantiomer of one each of these compounds (4- and 6- are enantiomers, as are 1- and 3-).

Phytase Specificity

5 As described above, phytases are divided according to their specificity in the initial hydrolysis step according to which phosphate-ester group is hydrolyzed first. Furthermore, plant phytases are generally 6-phytases. However, the lilly pollen phytase is said to be a 5-phytase. Microorganism derived phytases are generally 3-phytases (*e.g.*, 3-phytases have been derived from *Aspergillus*
10 *awamori* (strain ALK0243) and *Aspergillus niger* (strain NRRL 3135) (Gene 133:55-62, 1993, and Gene 127:87-94, 1993). The specificity of a phytase can be determined in several ways, *e.g.*, by HPLC or by NMR spectroscopy.

An isolate nucleic acid sequences encoding a phytase enzyme disclosed in
15 FIG. 1 (SEQ ID NO:1) can be used in the compositions of the invention or to produce a polypeptide for use in the compositions of the invention. In addition, isolated nucleic acid sequences that are substantially similar are provided if: (i) they are capable of hybridizing under stringent conditions, hereinafter described, to SEQ ID NO: 1; or (ii) the DNA sequences are degenerate to SEQ ID NO:1.
20 Degenerate DNA sequences encode the amino acid sequence of SEQ ID NO:2, but have variations in the nucleotide coding sequences. As used herein, "substantially similar" refers to the sequences having similar identity to the sequences of the instant invention. The nucleotide sequences that are substantially similar can be identified by hybridization or by sequence comparison (as
25 discussed above). Enzyme sequences that are substantially similar can also be identified by one or more of the following: proteolytic digestion, gel electrophoresis and/or microsequencing.

One means for isolating a nucleic acid molecule encoding a phytase
30 enzyme is to probe a genomic gene library with a natural or artificially designed probe using art recognized procedures (see, for example: Current Protocols in Molecular Biology, Ausubel F. M. *et al.* (EDS.) Green Publishing Company

Assoc. and John Wiley Interscience, New York, 1989, 1992). It is appreciated to one skilled in the art that SEQ ID NO:1, or fragments thereof (comprising at least 15 contiguous nucleotides), is a particularly useful probe. Other particular useful probes for this purpose are hybridizable fragments to the sequences of SEQ ID
5 NO:1 (*i.e.*, comprising at least 15 contiguous nucleotides).

With respect to nucleic acid sequences which hybridize to specific nucleic acid sequences disclosed herein, hybridization may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions.
10 As an example of oligonucleotide hybridization, a polymer membrane containing immobilized denatured nucleic acid is first prehybridized for 30 minutes at 45 °C. in a solution consisting of 0.9M NaCl, 50 mM NaH₂ PO₄, pH 7.0, 5.0 mM Na₂ EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately 2X 10⁷ cpm (specific activity 4-9 x 10⁸ cpm/μg) of ³²P end-
15 labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1 X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na₂ EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T_m-10 °C for the oligo-nucleotide probe. The membrane is then exposed to auto-
20 radiographic film for detection of hybridization signals.

Stringent conditions means hybridization will occur if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. See J. Sambrook *et al.*, Molecular Cloning, A Laboratory
25 Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory) which is hereby incorporated by reference in its entirety.

The polynucleotides are also useful for designing or mutational analysis in order to obtain enzymes having phytase activity but exhibit one or more
30 characteristics that are different from the original or native sequence. Such methodologies are discussed above. Accordingly, it is possible to obtain a phytase enzyme which is resistant to proteases, has higher activity, or increase

stability, using the polynucleotide and polypeptide sequences as indicated in SEQ ID Nos: 1 and 2, respectively. The invention, thus encompasses polynucleotides which differ from the reference polynucleotide such that the changes are silent changes, for example the changes do not alter the amino acid sequence encoded
5 by the polynucleotide, and also include changes which increase or modify the activity of the enzyme. The invention also relates to nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the enzyme encoded by the reference polynucleotide (SEQ ID NO:1). In a preferred aspect of the invention these enzymes retain the same biological action
10 as the enzyme encoded by the reference polynucleotide.

Provided is a substantially pure phytase enzyme that can be used in the compositions of the invention. The term "substantially pure" is used herein to describe a molecule, such as a polypeptide (*e.g.*, a phytase polypeptide, or a
15 fragment thereof) that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. The purity of the polypeptides can be determined using standard methods including, *e.g.*, polyacrylamide gel
20 electrophoresis (*e.g.*, SDS-PAGE), column chromatography (*e.g.*, high performance liquid chromatography (HPLC)), and amino-terminal amino acid sequence analysis.

The phytase polypeptide included in the invention can have the amino acid
25 sequences of a phytase shown in FIG. 1 (SEQ ID NO:2). Phytase polypeptides, such as those isolated from *E. coli* B, can be characterized by catalyzing the hydrolysis of phytate to inositol and free phosphate with the release of minerals from the phytic acid complex.

30 Also included for use in the compositions of the invention are polypeptides having sequences that are "substantially identical" to the sequence of a phytase polypeptide, such as the sequence set forth in SEQ ID NO:2. A "substantially

identical” amino acid sequence is a sequence that differs from a reference sequence by (1) conservative amino acid substitutions, for example, substitutions of one amino acid for another of the same class (*e.g.*, substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine) or (2) changes in the amino acid sequence that result in a polypeptide that has phytase activity but the activity is increased, decreased or the stability or resistance to proteases has been modified.

Fragments of a phytase polypeptide can also be used in the compositions of the invention so long as they retain at least one phytase-specific activity or epitope. Phytase activity can be assayed by examining the catalysis of phytate to inositol and free phosphate. For example, a phytase polypeptide fragment containing, *e.g.*, at least 8-10 amino acids can be used as an immunogen in the production of phytase-specific antibodies. The fragment can contain, for example, an amino acid sequence that is conserved in phytases, and this amino acid sequence can contain amino acids that are conserved in phytases. Such fragments can easily be identified by comparing the sequences of phytases found in FIG. 1.

Phytase polypeptides for use in the invention can be obtained using any of several standard methods. For example, phytase polypeptides can be produced in a standard recombinant expression systems (as described herein), chemically synthesized, or purified from an organism in which they are naturally expressed. The purified polypeptides can then be impregnated, incorporated or used in combination with the compositions of the invention (*e.g.*, a dietary aid of the invention). A polypeptide used with the compositions of the invention include for example, enzymes including truncated forms of phytase, and variants such as deletion and insertion variants.

Examples of such assays used to measure phytase activity include the following assay: Phytase activity can be measured by incubating 150 μ l of the

enzyme preparation with 600 μ l of 2 mM sodium phytate in 100 mM Tris HCl buffer pH 7.5, supplemented with 1 mM CaCl₂ for 30 minutes at 37 °C. After incubation the reaction is stopped by adding 750 μ l of 5% trichloroacetic acid. Phosphate released was measured against phosphate standard spectrophotometrically at 700 nm after adding 1500 μ l of the color reagent (4 volumes of 1.5% ammonium molybdate in 5.5% sulfuric acid and 1 volume of 2.7% ferrous sulfate; Shimizu, M., Biosci. Biotech. Biochem., 56:1266-1269, 1992). One unit of enzyme activity is defined as the amount of enzyme required to liberate one μ mol Pi per min under assay conditions. Specific activity can be expressed in units of enzyme activity per mg of protein.

A polynucleotide which encodes a mature enzyme, such as the phytase enzyme of FIG. 1 (*e.g.*, SEQ ID NO:2) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature enzyme (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

Variants of a phytase polynucleotide, which encode for analogs and derivatives of the enzyme having the deduced amino acid sequence of FIG. 1 (*e.g.*, SEQ ID NO:2), can also be used in the compositions of the invention. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, polynucleotides encoding the same mature enzyme as shown in FIG. 1 as well as variants of such polynucleotides which variants encode for a derivative or analog of the enzyme of FIG. 1 can be used with the compositions of the invention or the products encoded by the polynucleotides can be used in the compositions of the invention. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in FIG.

1. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more
5 nucleotides, which does not substantially alter the function of the encoded enzyme.

Polynucleotides, wherein the coding sequence for the mature enzyme may be fused in the same reading frame to a polynucleotide sequence which aids in
10 expression and secretion of an enzyme from a host cell, for example, a leader sequence which functions to control transport of an enzyme from the cell can also be used with the compositions of the invention. The enzyme having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the enzyme. The polynucleotides may also encode
15 for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains. Thus, for example, the polynucleotide may encode for a mature enzyme, or for an enzyme having a prosequence or for an enzyme having both a
20 prosequence and a presequence (leader sequence).

The invention further relates to an enzyme which has the deduced amino acid sequence of FIG. 1, as well as analogs and derivatives of such enzyme. Such enzymes can be used in the compositions of the invention.

25 The terms "derivative" and "analog" when referring to the enzyme of FIG. 1 means a enzyme which retains essentially the same biological function or activity as such enzyme. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature
30 enzyme.

The derivative or analog of the enzyme of FIG. 1 may be derived as described herein and includes (i) one in which one or more of the amino acid residues are substituted with an amino acid residue which is not encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes
5 a substituent group, or (iii) one in which the mature enzyme is fused with another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for purification of the mature enzyme or a proprotein
10 sequence. Such derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein. A variant, *i.e.* a “analog” or “derivative” enzyme, and reference enzyme may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

15

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography,
20 hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

25

The enzyme of this invention may be employed for any purpose in which such enzyme activity is necessary or desired. In a preferred embodiment the enzyme is employed for catalyzing the hydrolysis of phytate. The degradation of phytate may be used in animal feed and the compositions of the invention.

30

In a preferred embodiment, the enzyme used in the compositions (*e.g.*, a dietary aid) of the present invention is a phytase enzyme which is stable to heat and is heat resistant and catalyzes the enzymatic hydrolysis of phytate, *i.e.*, the

enzyme is able to renature and regain activity after a brief (*i.e.*, 5 to 30 seconds), or longer period, for example, minutes or hours, exposure to temperatures of above 50 °C.

5 A “feed” and a “food,” respectively, means any natural or artificial diet, meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by an animal and a human being, respectively. “Dietary Aid,” as used herein, denotes, for example, a composition containing agents that provide a therapeutic or digestive agent to an animal or organism. A “dietary aid,”
10 typically is not a source of caloric intake for an organism, in other words, a dietary aid typically is not a source of energy for the organism, but rather is a composition which is taken in addition to typical “feed” or “food”.

 An agent or enzyme (*e.g.*, a phytase) may exert its effect *in vitro* or *in vivo*,
15 *i.e.* before intake or in the stomach or gizzard of the organism, respectively. Also a combined action is possible.

 Although any enzyme may be incorporated into a dietary aid, reference is made herein to phytase as an exemplification of the methods and compositions of
20 the invention. A dietary aid of the invention includes an enzyme (*e.g.*, a phytase). Generally, a dietary aid containing a phytase composition is liquid or dry.

 Liquid compositions need not contain anything more than the enzyme (*e.g.* a phytase), preferably in a highly purified form. Usually, however, a stabilizer
25 such as glycerol, sorbitol or mono propylen glycol is also added. The liquid composition may also comprise other additives, such as salts, sugars, preservatives, pH-adjusting agents, proteins, phytate (a phytase substrate). Typical liquid compositions are aqueous or oil-based slurries. The liquid compositions can be added to a biocompatible composition for slow release. Preferably the enzyme
30 is added to a dietary aid composition that is a biocompatible material (*e.g.*, biodegradable or non-biodegradable) and includes the addition of recombinant cells into, for example, porous microbeads.

Dry compositions may be spray dried compositions, in which case the composition need not contain anything more than the enzyme in a dry form. Usually, however, dry compositions are so-called granulates which may readily be
5 mixed with a food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is compatible with that of the other components of the mixture. This provides a safe and convenient means of incorporating enzymes into animal feed. Preferably the granulates are biocompatible and more preferably they biocompatible granulates are non-
10 biodegradable.

Agglomeration granulates coated by an enzyme can be prepared using agglomeration technique in a high shear mixer. Absorption granulates are prepared by having cores of a carrier material to absorb/be coated by the enzyme.
15 Preferably the carrier material is a biocompatible non-biodegradable material that simulates the role of stones or grit in the gizzard of an animal. Typical filler materials used in agglomeration techniques include salts, such as disodium sulphate. Other fillers are kaolin, talc, magnesium aluminium silicate and cellulose fibres. Optionally, binders such as dextrans are also included in
20 agglomeration granulates. The carrier materials can be any biocompatible material including biodegradable and non-biodegradable materials (*e.g.*, rocks, stones, ceramics, various polymers). Optionally, the granulates are coated with a coating mixture. Such mixture comprises coating agents, preferably hydrophobic coating agents, such as hydrogenated palm oil and beef tallow, and if desired other
25 additives, such as calcium carbonate or kaolin.

Additionally, the dietary aid compositions (*e.g.*, phytase dietary aid compositions) may contain other substituents such as colouring agents, aroma compounds, stabilizers, vitamins, minerals, other feed or food enhancing enzymes
30 etc. A typical additive usually comprises one or more compounds such as vitamins, minerals or feed enhancing enzymes and suitable carriers and/or excipients.

In a one embodiment, the dietary aid compositions of the invention additionally comprises an effective amount of one or more feed enhancing enzymes, in particular feed enhancing enzymes selected from the group consisting of α -galactosidases, β -galactosidases, in particular lactases, other phytases, β -glucanases, in particular endo- β -1,4-glucanases and endo- β -1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4- β -galactosidases and arabinogalactan endo-1,3- β -galactosidases, endoglucanases, in particular endo-1,2- β -glucanase, endo-1,3- α -glucanase, and endo-1,3- β -glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan- α -rhamnosidase, pectate lyases, and α -galacturonisidases, mannanases, β -mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases.

The animal dietary aid of the invention is supplemented to the mono-gastric animal before or simultaneously with the diet. In one embodiment, the dietary aid of the invention is supplemented to the mono-gastric animal simultaneously with the diet. In another embodiment, the dietary aid is added to the diet in the form of a granulate or a stabilized liquid.

An effective amount of an enzyme in a dietary aid of the invention is from about 10-20,000; preferably from about 10 to 15,000, more preferably from about 10 to 10,000, in particular from about 100 to 5,000, especially from about 100 to about 2,000 FYT/kg dietary aid.

Examples of other specific uses of the phytase of the invention is in soy processing and in the manufacture of inositol or derivatives thereof.

30

The invention also relates to a method for reducing phytate levels in animal manure, wherein the animal is fed a dietary aid containing an effective

amount of the phytase of the invention. As stated in the beginning of the present application one important effect thereof is to reduce the phosphate pollution of the environment.

5 In another embodiment, the dietary aid is a magnetic carrier. For example, a magnetic carrier containing an enzyme (*e.g.*, a phytase) distributed in, on or through a magnetic carrier (*e.g.*, a porous magnetic bead), can be distributed over an area high in phytate and collected by magnets after a period of time. Such distribution and recollection of beads reduces additional pollution and allows for
10 reuse of the beads. In addition, use of such magnetic beads *in vivo* allows for the localization of the dietary aid to a point in the digestive tract where, for example, phytase activity can be carried out. For example, a dietary aid of the invention containing digestive enzymes (*e.g.*, a phytase) can be localized to the gizzard of the animal by juxtapositioning a magnet next to the gizzard of the animal after the
15 animal consumes a dietary aid of magnetic carriers. The magnet can be removed after a period of time allowing the dietary aid to pass through the digestive tract. In addition, the magnetic carrier is useful to remove the biocompatible substance from animals used as foodstuff upon sacrificing or for collection of the biocompatible substance.

20

 When the dietary aid is a porous particle, such particles are typically impregnated by a substance with which it is desired to release slowly to form a slow release particle. Such slow release particles may be prepared not only by impregnating the porous particles with the substance it is desired to release, but
25 also by first dissolving the desired substance in the first dispersion phase. In this case, slow release particles prepared by the method in which the substance to be released is first dissolved in the first dispersion phase are also within the scope and spirit of the invention. The porous hollow particles may, for example, be impregnated by a slow release substance such as a medicine, agricultural chemical
30 or enzyme. In particular, when porous hollow particles impregnated by an enzyme are made of a biodegradable polymers, the particles themselves may be used as an

agricultural chemical or fertilizer, and they have no adverse effect on the environment. In one embodiment the porous particles are magnetic in nature.

The porous hollow particles may be used as a bioreactor support, in particular an enzyme support. Therefore, it is advantageous to prepare the dietary aid utilizing a method of a slow release, for instance by encapsulating the enzyme of agent in a microvesicle, such as a liposome, from which the dose is released over the course of several days, preferably between about 3 to 20 days. Alternatively, the agent (*e.g.*, an enzyme) can be formulated for slow release, such as incorporation into a slow release polymer from which the dosage of agent (*e.g.*, enzyme) is slowly released over the course of several days, for example from 2 to 30 days and can range up to the life of the animal.

As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multilamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain stabilizers, preservatives, excipients, and the like in addition to the agent. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., *Methods in Cell Biology*, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 et seq.

Also within the scope of the invention is the use of a phytase of the invention during the preparation of food or feed preparations or additives, *i.e.*, the phytase exerts its phytase activity during the manufacture only and is not active in the final food or feed product. This aspect is relevant for instance in dough making and baking. Accordingly, phytase or recombinant yeast expressing phytase can be impregnated in, on or through a magnetic carriers, distributed in the dough or food medium, and retrieved by magnets.

The dietary aid of the invention may be administered alone to animals in an biocompatible (*e.g.*, a biodegradable or non-biodegradable) carrier or in combination with other digestion additive agents. The dietary aid of the invention thereof can be readily administered as a top dressing or by mixing them directly
5 into animal feed or provided separate from the feed, by separate oral dosage, by injection or by transdermal means or in combination with other growth related edible compounds, the proportions of each of the compounds in the combination being dependent upon the particular organism or problem being addressed and the degree of response desired. It should be understood that the specific dietary
10 dosage administered in any given case will be adjusted in accordance with the specific compounds being administered, the problem to be treated, the condition of the subject and the other relevant facts that may modify the activity of the effective ingredient or the response of the subject, as is well known by those skilled in the art. In general, either a single daily dose or divided daily dosages
15 may be employed, as is well known in the art.

If administered separately from the animal feed, forms of the dietary aid can be prepared by combining them with non-toxic pharmaceutically acceptable edible carriers to make either immediate release or slow release formulations, as is
20 well known in the art. Such edible carriers may be either solid or liquid such as, for example, corn starch, lactose, sucrose, soy flakes, peanut oil, olive oil, sesame oil and propylene glycol. If a solid carrier is used the dosage form of the compounds may be tablets, capsules, powders, troches or lozenges or top dressing as micro-dispersable forms. If a liquid carrier is used, soft gelatin capsules, or
25 syrup or liquid suspensions, emulsions or solutions may be the dosage form. The dosage forms may also contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, etc. They may also contain other therapeutically valuable substances.

30 Thus, a significant advantages of the invention include for example, 1) ease of manufacture of the active ingredient loaded biocompatible compositions; 2) versatility as it relates to the class of polymers and/or active ingredients which

may be utilized; 3) higher yields and loading efficiencies; and 4) the provision of sustained release formulations that release active, intact active agents *in vivo*, thus providing for controlled release of an active agent over an extended period of time. In addition, another advantage is due to the local delivery of the agent with
5 in the digestive tract (*e.g.*, the gizzard) of the organism. As used herein the phrase “contained within” denotes a method for formulating an agent into a composition useful for controlled release, over an extended period of time of the agent.

In the sustained-release or slow release compositions of the invention, an
10 effective amount of an agent (*e.g.*, an enzyme or antibiotic) will be utilized. As used herein, sustained release or slow release refers to the gradual release of an agent from a biocompatible material, over an extended period of time. The sustained release can be continuous or discontinuous, linear or non-linear, and this can be accomplished using one or more biodegradable or non-biodegradable
15 compositions, drug loadings, selection of excipients, or other modifications. However, it is to be recognized that it may be desirable to provide for a “fast” release composition, that provides for rapid release once consumed by the organism. It is also to be understood that by “release” does not necessarily mean that the agent is released from the biocompatible carrier. Rather in one
20 embodiment, the slow release encompasses slow activation or continual activation of an agent present on the biocompatible composition. For example, a phytase need not be released from the biocompatible composition to be effective. In this embodiment, the phytase is immobilized on the biocompatible composition.

25 The animal feed may be any protein-containing organic meal normally employed to meet the dietary requirements of animals. Many of such protein-containing meals are typically primarily composed of corn, soybean meal or a corn/soybean meal mix. For example, typical commercially available products fed to fowl include Egg Maker Complete, a poultry feed product of Land O’Lakes AG
30 Services, as well as Country Game & Turkey Grower a product of Agwa, Inc. (see also The Emu Farmer’s Handbook by Phillip Minnaar and Maria Minnaar). Both of these commercially available products are typical examples of animal feeds

with which the present dietary aid and/or the enzyme phytase may be incorporated to reduce or eliminate the amount of supplemental phosphorus, zinc, manganese and iron intake required in such compositions.

5 The present invention is applicable to the diet of numerous animals, which herein is defined as including mammals (including humans), fowl and fish. In particular, the diet may be employed with commercially significant mammals such as pigs, cattle, sheep, goats, laboratory rodents (rats, mice, hamsters and gerbils), fur-bearing animals such as mink and fox, and zoo animals such as
10 monkeys and apes, as well as domestic mammals such as cats and dogs. Typical commercially significant avian species include chickens, turkeys, ducks, geese, pheasants, emu, ostrich, loons, kiwi, doves, parrots, cockatiel, cockatoo, canaries, penguins, flamingoes, and quail. Commercially farmed fish such as trout would also benefit from the dietary aids disclosed herein. Other fish that can benefit
15 include, for example, fish (especially in an aquarium or aquaculture environment, e.g., tropical fish), goldfish and other ornamental carp, catfish, trout, salmon, shark, ray, flounder, sole, tilapia, medaka, guppy, molly, platyfish, swordtail, zebrafish, and loach.

20 While the procedures described in the examples are typical of those that can be used to carry out certain aspects of the invention, other procedures known to those skilled in the art can also be used. Accordingly, the following examples should not be construed to limit the invention.

25

EXAMPLE 1

Isolation, Bacterial Expression and Purification of Phytase

E.coli B genomic DNA was obtained from Sigma (Catalog # D-2001), St. Louis, N.J.

30

The following primers were used to PCR amplify the gene directly from the genomic DNA:

5' primer gtttctgaattcaaggaggaatttaaATGAAAGCGATCTTAATCCCATT
(SEQ ID NO:3)

3' primer gtttctggatccTTACAAACTGCACGCCGGTAT (SEQ ID NO:4)

5

Pfu polymerase in the PCR reaction, and amplification was performed according to manufacturers protocol (Stratagene Cloning Systems, Inc., La Jolla, Calif.).

10

PCR product was purified and the purified product and pQE60 vector (Qiagen) were both digested with EcoRI and BglII restriction endonucleases (New England Biolabs) according to manufacturers protocols. Overnight ligations were performed using standard protocols to yield pQE60.

15

The amplified sequences were inserted in frame with the sequence encoding for the RBS. The ligation mixture was then used to transform the *E. coli* strain M15/pREP4 (Qiagen, Inc.) by electroporation. M15/pREP4 contains multiple copies of the plasmid pREP4, which expresses the *lacI* repressor and also confers kanamycin resistance (Kan^r). Plasmid DNA was isolated and confirmed
20 by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.⁶⁰⁰) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside")
25 was then added to a final concentration of 1 mM. IPTG induces by inactivating the *lacI* repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation.

30

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described. It is to be understood that, while the invention has been described with

reference to the above detailed description, the foregoing description is intended to illustrate, but not to limit, the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the following claims.

All publications, patent applications, patents, and other references mentioned
5 herein are incorporated by reference in their entirety.

SEQUENCE LISTING

<110> Short, Jay M.
Kretz, Keith
O'Donoghue, Eileen

<120> DIETARY AIDS AND METHODS OF USE THEREOF

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PCT/US01/15764

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33

WHAT IS CLAIMED IS:

1. A dietary aid, comprising:
a sustained release biocompatible composition comprising an agent that assists in digestion, wherein the biocompatible composition is effective upon oral consumption and release in the digestive tract of a subject.
2. The dietary aid of claim 1, wherein the agent is an enzyme.
3. The dietary aid of claim 2, wherein the enzyme is selected from the group consisting of a phytase, an amylase, an esterase, a cellulase, an α -galactosidase, a β -galactosidase, a β -glucanases, a xylosidase, a galactanase, an endoglucanase, a pectin degrading enzyme, a polygalacturonase, an arabinanase, a rhamnogalacturonase, a rhamnogalacturonan acetyl esterase, a rhamnogalacturonan- α -rhamnosidase, a pectate lyase, an α -galacturonisidases, a mannanase, β -mannosidase, a mannan acetyl esterase, a xylan acetyl esterase, a protease, an arabinoxylanase, and a xylanase.
4. The dietary aid of claim 3, wherein the phytase is selected from the group consisting of a 1-phytase, a 2-phytase, a 3-phytase, a 4-phytase, a 5-phytase and a 6-phytase.
5. The dietary aid of claim 3, wherein the phytase has a sequence as set forth in SEQ ID NO:2 and variants or fragments thereof.
6. The dietary aid of claim 2, wherein the enzyme is modified to increase the enzymes activity, stability, or resistance to protease degradation of pH inactivation.

7. The dietary aid of claim 6, wherein the modifications are introduced by a method selected from the group consisting of error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, ligation reassembly, GSSM and any combination thereof.
8. The dietary aid of claim 1, wherein the agent is an antibiotic.
9. The dietary aid of claim 1, wherein the agent is a hormone.
10. The dietary aid of claim 1, wherein the biocompatible composition is biodegradable.
11. The dietary aid of claim 10, wherein the biodegradable biocompatible composition is selected from the group consisting of poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides, polycarbonate, polycyanoacrylate, polyurethanes, polyacrylate, blends, copolymers and combinations thereof.
12. The dietary aid of claim 1, wherein the biocompatible composition is non-biodegradable.
13. The dietary aid of claim 12, wherein the non-biodegradable composition is a rock, stone, glass, metal or ceramic composition.
14. The dietary aid of claim 13, wherein the composition is porous.
15. The dietary aid of claim 12, wherein the composition is magnetic.
16. The dietary aid of claim 15, wherein the magnetic composition is porous.

17. The dietary aid of claim 12, wherein the non-biodegradable composition is selected from the group consisting of acrylic, modacrylic, polyamide, polycarbonate, polyester, polyethylene, polypropylene, polystyrene, polysulfone, polyethersulfone, polyvinylidene fluoride polyurethane, polyvinyl alcohol and silicone.
18. The dietary aid of claim 1, wherein the organism is a canine, feline, porcine, equine, or bovine species.
19. The dietary aid of claim 1, wherein the subject is a fowl.
20. The dietary aid of claim 19, wherein the fowl is an ostrich, chicken, rooster, quail, dove, pigeon, or parrot.
21. The dietary aid of claim 1, wherein the subject is a fish.
22. The dietary aid of claim 21, wherein the fish is selected from the group consisting of goldfish, carp, catfish, trout, salmon, shark, ray, flounder, sole, tilapia, medaka, guppy, molly, platyfish, swordtail, zebrafish, and loach.
23. A method of aiding digestion of an organism, comprising:
delivering to the digestive tract of the organism a biocompatible composition for sustained release of an agent that assists in digestion upon oral consumption, wherein release of the agent results in aiding digestion of the organism.
24. The method of claim 23, wherein the agent is an enzyme.

25. The method of claim 24, wherein the enzyme is selected from the group consisting of a phytase, an amylase, an esterase, a cellulase, an α -galactosidase, a β -galactosidase, a β -glucanases, a xylosidase, a galactanase, an endoglucanase, a pectin degrading enzyme, a polygalacturonase, an arabinanase, a rhamnogalacturonase, a rhamnogalacturonan acetyl esterase, a rhamnogalacturonan- α -rhamnosidase, a pectate lyase, an α -galacturonisidases, a mannanase, β -mannosidase, a mannan acetyl esterase, a xylan acetyl esterase, a protease, an arabinoxylanase, and a xylanase.
26. The method of claim 25, wherein the phytase is selected from the group consisting of a 1-phytase, a 2-phytase, a 3-phytase, a 4-phytase, a 5-phytase and a 6-phytase.
27. The method of claim 25, wherein the phytase has a sequence as set forth in SEQ ID NO:2 and variants or fragments thereof.
28. The method of claim 24, wherein the enzyme is modified to increase the enzymes activity, stability, or resistance to protease degradation of pH inactivation.

29. The method of claim 28, wherein the modifications are introduced by a method selected from the group consisting of error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, ligation reassembly, GSSM and any combination thereof.
30. The method of claim 23, wherein the agent is an antibiotic.
31. The method of claim 23, wherein the agent is a hormone.
32. The method of claim 23, wherein the biocompatible composition is biodegradable.
33. The method of claim 32, wherein the biodegradable biocompatible composition is selected from the group consisting of poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides, polycarbonate, polycyanoacrylate, polyurethanes, polyacrylate, blends, copolymers and combinations thereof.
34. The method of claim 23, wherein the biocompatible composition is non-biodegradable.
35. The method of claim 34, wherein the non-biodegradable composition is a rock, stone, glass, metal or ceramic composition.
36. The method of claim 35, wherein the composition is porous.
37. The method of claim 34, wherein the composition is magnetic.
38. The method of claim 37, wherein the magnetic composition is porous.

39. The method of claim 34, wherein the non-biodegradable composition is selected from the group consisting of acrylic, modacrylic, polyamide, polycarbonate, polyester, polyethylene, polypropylene, polystyrene, polysulfone, polyethersulfone, polyvinylidene fluoride polyurethane, polyvinyl alcohol and silicone.
40. The method of claim 23, wherein the organism is a canine, feline, porcine, equine, or bovine species.
41. The method of claim 23, wherein the organism is a fowl.
42. The method of claim 41, wherein the fowl is an ostrich, chicken, rooster, quail, dove, pigeon, or parrot.
43. The method of claim 23, wherein the organism is a fish.
44. The method of claim 43, wherein the fish is selected from the group consisting of goldfish, carp, catfish, trout, salmon, shark, ray, flounder, sole, tilapia, medaka, guppy, molly, platyfish, swordtail, zebrafish, and loach.
45. The method of claim 23, wherein the digestive tract includes a gizzard.
46. The method of claim 37, wherein the composition is localized to an area of the digestive tract by use of a magnetic external to the digestive tract.
47. The method of claim 46, wherein the composition is localized to a gizzard of the digestive tract.

FIGURE 1 a

(SEQ ID NO:1-nucleotide sequence and SEQ ID NO:2-amino acid sequence)

Escherichia coli B Phytase Sequence

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      1
      ATG AAA GCG ATC TTA ATC CCA TTT TTA TCT CTT CTG ATT CCG
TTA ACC CCG
      Met Lys Ala Ile Leu Ile Pro Phe Leu Ser Leu Leu Ile Pro
Leu Thr Pro

      CAA TCT GCA TTC GCT CAG AGT GAG CCG GAG CTG AAG CTG GAA
AGT GTG GTG
      Gln Ser Ala Phe Ala Gln Ser Glu Pro Glu Leu Lys Leu Glu
Ser Val Val

      ATT GTC AGT CGT CAT GGT GTG CGT GCT CCA ACC AAG GCC ACG
CAA CTG ATG
      Ile Val Ser Arg His Gly Val Arg Ala Pro Thr Lys Ala Thr
Gln Leu Met

      CAG GAT GTC ACC CCA GAC GCA TGG CCA ACC TGG CCG GTA AAA
CTG GGT TGG
      Gln Asp Val Thr Pro Asp Ala Trp Pro Thr Trp Pro Val Lys
Leu Gly Trp

      CTG ACA CCG CGN GGT GGT GAG CTA ATC GCC TAT CTC GGA CAT
TAC CAA CGC
      Leu Thr Pro Arg Gly Gly Glu Leu Ile Ala Tyr Leu Gly His
Tyr Gln Arg

      CAG CGT CTG GTA GCC GAC GGA TTG CTG GCG AAA AAG GGC TGC
CCG CAG TCT
      Gln Arg Leu Val Ala Asp Gly Leu Leu Ala Lys Lys Gly Cys
Pro Gln Ser

      GGT CAG GTC GCG ATT ATT GCT GAT GTC GAC GAG CGT ACC CGT
AAA ACA GGC
      Gly Gln Val Ala Ile Ile Ala Asp Val Asp Glu Arg Thr Arg
Lys Thr Gly

      GAA GCC TTC GCC GCC GGG CTG GCA CCT GAC TGT GCA ATA ACC
GTA CAT ACC

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FIGURE 1b

Val His Thr Glu Ala Phe Ala Ala Gly Leu Ala Pro Asp Cys Ala Ile Thr
 AAA ACT GGC CAG GCA GAT ACG TCC AGT CCC GAT CCG TTA TTT AAT CCT CTA
 Lys Thr Gly Gln Ala Asp Thr Ser Ser Pro Asp Pro Leu Phe Asn Pro Leu
 AGC AGG GCA GTT TGC CAA CTG GAT AAC GCG AAC GTG ACT GAC GCG ATC CTC
 Ser Arg Ala Val Cys Gln Leu Asp Asn Ala Asn Val Thr Asp Ala Ile Leu
 TTT CGC GAA GGA GGG TCA ATT GCT GAC TTT ACC GGG CAT CGG CAA ACG GCG
 Phe Arg Glu Gly Gly Ser Ile Ala Asp Phe Thr Gly His Arg Gln Thr Ala
 AAA CGT GAG CTG GAA CGG GTG CTT AAT TTT CCG CAA TCA AAC TTG TGC CTT
 Lys Arg Glu Leu Glu Arg Val Leu Asn Phe Pro Gln Ser Asn Leu Cys Leu
 GAA CTC AAG AAA CAG GAC GAA AGC TGT TCA TTA ACG CAG GCA TTA CCA TCG
 Glu Leu Lys Lys Gln Asp Glu Ser Cys Ser Leu Thr Gln Ala Leu Pro Ser
 GCA TCA ATG GTG AGC GCC GAC AAT GTC TCA TTA ACC GGT GCG GTA AGC CTC
 Ala Ser Met Val Ser Ala Asp Asn Val Ser Leu Thr Gly Ala Val Ser Leu
 GAG CCG GGG CTG ACG GAG ATA TTT CTC CTG CAA CAA GCA CAG GGA ATG CCG
 Glu Pro Gly Leu Thr Glu Ile Phe Leu Leu Gln Gln Ala Gln Gly Met Pro
 AGT TTG CAT TGG GGA AGG ATC ACC GAT TCA CAC CAG TGG AAC ACC TTG CTA
 Ser Leu His Trp Gly Arg Ile Thr Asp Ser His Gln Trp Asn Thr Leu Leu
 CGC AGC CGC AAC GCG CAA TTT TAT TTG CTA CAA CGC ACG CCA GAG GTT GCC
 Arg Ser Arg Asn Ala Gln Phe Tyr Leu Leu Gln Arg Thr Pro Glu Val Ala

FIGURE 1c

GCC ACC CCG TTA TTG GAT TTG ATC ATG GCA GCG TTG ACG CCC
 CAT CCA CCG
 Ala Thr Pro Leu Leu Asp Leu Ile Met Ala Ala Leu Thr Pro
 His Pro Pro

CAA AAA CAG GCG TAT GGT GTG ACA TTA CCC ACT TCA GTA CTG
 TTT ATT GCC
 Gln Lys Gln Ala Tyr Gly Val Thr Leu Pro Thr Ser Val Leu
 Phe Ile Ala

GGA CAC GAT ACT AAT CTG GCA AAT CTC GGC GGC GCA CTG GAG
 CTC AAC TGG
 Gly His Asp Thr Asn Leu Ala Asn Leu Gly Gly Ala Leu Glu
 Leu Asn Trp

ACG CTT CCC GGT CAG CCG GAT AAC ACG CCG CCA GGT GGT GAA
 CTG GTG TTT
 Thr Leu Pro Gly Gln Pro Asp Asn Thr Pro Pro Gly Gly Glu
 Leu Val Phe

GAA CGC TGG CGT CGG CTA AGC GAT AAC AGC CAG TGG ATT CAG
 GTT TCG CTG
 Glu Arg Trp Arg Arg Leu Ser Asp Asn Ser Gln Trp Ile Gln
 Val Ser Leu

GTC TTC CAG ACT TTA CAG CAG ATG CGT GAT AAA ACG CCG CTG
 TCA TTA AAT
 Val Phe Gln Thr Leu Gln Gln Met Arg Asp Lys Thr Pro Leu
 Ser Leu Asn

ACG CCG CCC GGA GAG GTG AAA CTG ACC CTG GCA GGA TGT GAA
 GAG CGA AAT
 Thr Pro Pro Gly Glu Val Lys Leu Thr Leu Ala Gly Cys Glu
 Glu Arg Asn

GCG CAG GGC ATG TGT TCG TTG GCA GGT TTT ACG CAA ATC GTG
 AAT GAA GCA
 Ala Gln Gly Met Cys Ser Leu Ala Gly Phe Thr Gln Ile Val
 Asn Glu Ala

CGC ATA CCG GCG TGC AGT TTG AGA TCT CAT CAC CAT CAC CAT
 CAC TAA 1323
 Arg Ile Pro Ala Cys Ser Leu Arg Ser His His His His His
 His End

FIGURE 2
pH/Temperature Profile and Stability

