Materials & Methods

3. MATIRIALS AND METHOD

The experimental part in this study is classified to five main steps as follows:

- Surveying the case study area (Egyptian Starch, Yeast and Detergents Company) (E.S.Y.D) and water balance in the company.
- Wastewater sampling and analysis.
- Precipitation and separation of water-soluble proteins and other dry matter contents from wastewater.
- Analysis of the separated dry matter and its contents of proteins
- Biological analysis

3.1. Study area

Egyptian Starch, Yeast and Detergents Company is one of the subsidiaries of Food Industries Holding Company. The head office is located in Siof area in Alexandria Governorate, Egypt. The company includes 4 factories to produce detergents, yeast, chemicals and assistance substances and starch factory.

Starch factory only produces starch from broken rice in Egypt. Starch factory area is 2,000 m^2 within the factories compound in Siof area at east of Alexandria. The maximum capacity of the plant is 30-35 tons of starch and modified starch per day. The main products are various types of industrial starch, food starch, dextrin and modified starch for textiles and petroleum industry.

Starch factory in Egyptian Starch, Yeast and Detergent Company produces raw starch and its derivatives from broken rice; maximum capacity of using broken rice is 50 ton / day, the current operating rate is 10 tons of broken rice /day. Table (18) shows the production volumes and broken rice used in the period from 01/07/2005 to 30/06/2012 (Financial Sector, 2008: 2012) [(132)].

Table (18): Starch Production situation in E.S.Y.D

Period	Volume of Production Ton	Broken rice used Ton			
		Zero	One	Two	Total
From 01/07/2007 to 30/06/2008	1793	1878	131	210	2219
From 01/07/2008 to 30/06/2009	1619	1878	80	zero	1958
From 01/07/2009 to 30/06/2010	1394	1618	22	zero	1640
From 01/07/2010 to 30/06/2011	607	425	16	zero	441
From 01/07/2011 to 30/06/2012	1127	789	196	27	1012

Source:: (Financial Sector, 2008:2012)[(132)]

3.2. Production process

Starch is extracted from two sorts of broken rice (Zero - One) as the following production processes:

- Initially cleaning by clean water to remove dust and impurities,
- Then soaking in pools with a dilute solution of caustic soda 0.6 to 0.8% for 10: 12 h in the presence of a stream of compressed air for 30 min to stir to raise moisture grain and dissolve much of the protein, the soaking process is considered complete when the grains can be crushed when pressed between the fingers,
- The soaking wastewater discharge to the settling tank,
- Addition of water and/or very dilute sodium hydroxide to form starch milk. The starch milk is passed over rotary screens to remove fibers and coarse particles. Milling and screening processes are repeated several times to produce homogeneous starch milk,
- Fibers and all coarse after separating starch are diluted and discharge to settling tank,
- By centrifugation (1500 rpm), protein is separated from starch milk suspension into heavy face (starch) and light face (water + protein),
- The separated protein from the first stage discharged with water into settling tank.
- The heavy face (starch) passed through another centrifuge (Strakuza 900 rpm) and (Krauss 600 rpm) to starch sediment to form damp starch cake which contain about 45% moisture, by sharp knifes the starch cakes are divided into smaller cubes which are dried by two units, the first is (Botner dryer) where the wet starch is placed in aluminum boards and drying by hot air until the moisture decreased to 10-12%, the produced starch called crystal starch, the second is (Flash dryer) where the wet starch is carried by hot air through the dryer until the starch powder moisture decreased to 10-12%. Figure (18) represents a detailed flow diagram of the rice starch processing line.

Raw water used in the factory is obtained from Alexandria Drinking Water Company through Siof purification plant. The total raw water consumption in 2012 was nearly 10000 m^3/ month.

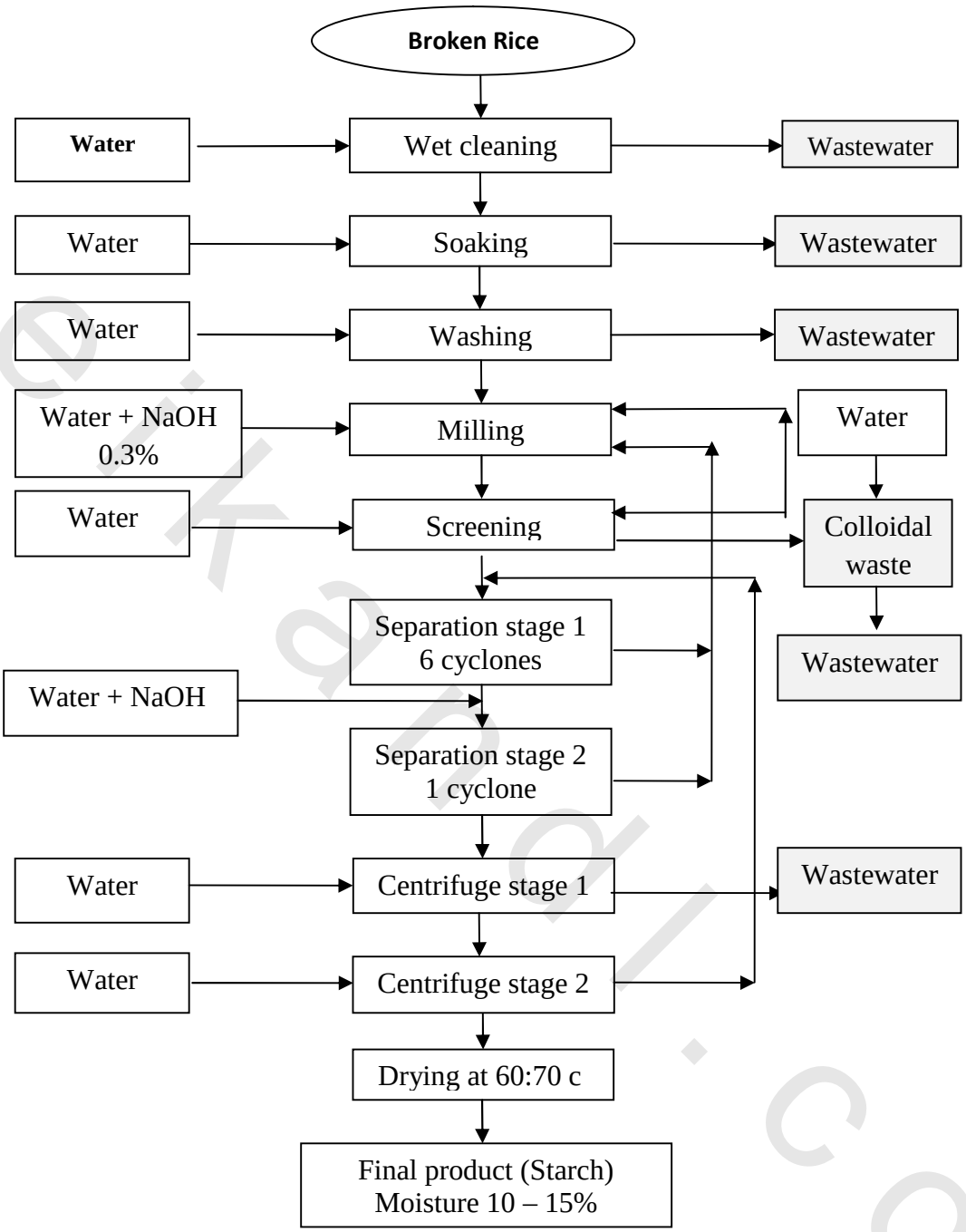

Figure (15): Rice starch production process at Egyptian Starch, Yeast and detergent company

3. 3. Water balance in the production process

Throughout the different manufacture processes, the quantities of water intake consumption were recorded through water meter which is installed in each process. Discharged wastewater was also measured using basket and stop watch.

3.4. Wastewater sampling

Wastewater samples were collected randomly from five discharge points and from settling tank before settling throughout 12 months through work duration. The five discharge points were:

1- Wet cleaning wastewater
2- Soaking wastewater
3- Washing wastewater
4- First centrifugal wastewater
5- Screen milling wastewater

Wastewater samples were preserved at 4˚C against fermentation in glass containers until analysis was performed.

3.5. Analysis of wastewater

Thirteen parameters were investigated by analysis of wastewater in "Environmental Monitoring and Quality Control Laboratories" in the Egyptian Starch, Yeast, and Detergents Company, according to **(**Standard Methods for the Examination of Water and Wastewater 20th Edition, 1998 and A.O.A.C, 2000)[133, 134].

These parameters are: pH, temperature, total solids, total suspended solids, total dissolved solids, chemical oxygen demand, biochemical oxygen demand, crude protein content, crude fat, total ash, crude fiber, carbohydrate content and sodium chloride.

3.5.1. pH

A pH meter (MODEL MI 151) is used to measure pH values of the wastewater samples, Figure 19.

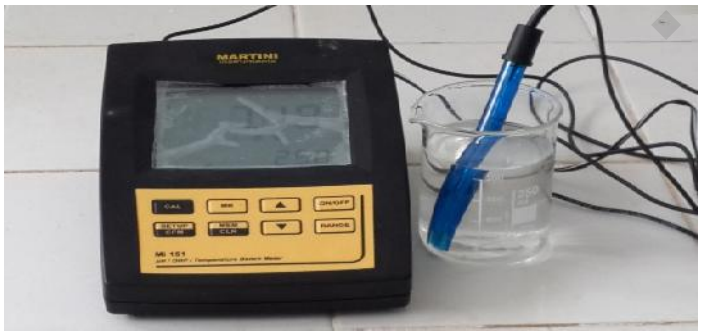

Figure (16): pH meter (MODEL MI 151)

3.5.2. Temperature

Temperature of the wastewater samples was measured by Celsius Thermometer

3.5.3. Total solids content (TS)

Total solids (TS), was determined in all the suspended, colloidal and dissolved solids in a sample of wastewater. Total solids were determined According to the method No.2540 B in Standard Methods for the Examination of Water and Wastewater 20th Edition [133] as follow:

A well-mixed sample is evaporated in a weighed dish and dried to constant weight in an oven at 103 to 105°C. The increase in weight over that of the empty dish represents the total solids according to the following equation

$$\text{Total solids (mg/l)} = \frac{(A - B) \text{ X } 1000}{\text{Sample volume (ml)}}$$

Where:
A = weight of dried residue + dish (mg), and
B = weight of dish (mg)

3.5.4. Total suspended solids (TSS)

TSS was determined according to the method No.2540 D in Standard Methods for the Examination of Water and Wastewater 20th Edition [133]. A well-mixed sample is filtered through a weighed standard glass-fiber filter and the residue retained on the filter is dried to a constant weight at 103 to 105°C. The increase in weight of the filter represents the total suspended solids according to the following equation.

$$\text{Total Suspended Solids (mg/l)} = \frac{(A - B) \text{ X } 1000}{\text{Sample volume (ml)}}$$

Where:
A = weight of filter + dried residue (mg), and
B = weight of filter (mg).

3.5.5. Total dissolved solids (TDS)

TDS was determined according to the method No.2540 C in Standard Methods for the Examination of Water and Wastewater 20th Edition [133]. A well-mixed sample is filtered through a standard glass fiber filter, and the filtrate is evaporated to dryness in weighed dish and dried to constant weight at 180 °C. The increase in dish weight represents the TDS according to the following equation:

$$\text{Total Dissolved Solids (mg/l)} = \frac{(A - B) \text{ X } 1000}{\text{Sample volume (ml)}}$$

Where:
A = weight of dried residue + dish (mg), and
B = weight of dish (mg).

3.5.6. Chemical oxygen demand (COD)

COD is used as a measure of oxygen equivalent to the organic matter content of sample that is susceptible to oxidation by a strong chemical oxidant.

COD was determined according to the method No.5220 in Standard Methods for the Examination of Water and Wastewater 20th Edition [133]. The closed reflux method was employed using potassium dichromate as the chemical oxidant. 50 ml of sample was taken into a refluxing flask and several boiling stones were added. 0.1 g HgSO4 was added to the solution. 5 ml of concentrated H_2SO_4 was also added to the solution. To ensure that $HgSO_4$ dissolved completely, the solution was swirled slowly while adding sulphuric acid. 0.1 g of Ag_2SO_4 was added to this solution. Finally potassium dichromate was added. Mixing the solution was ensured by swirling the flask in a water bath to recover any volatile substances that may have escaped from the liquid state. The flask was then attached to the condenser and further cooling was done. 20 ml of sulphuric acid was added to the solution in the flask, continuing cooling and swirling to mix the solution. The solution was refluxed for 1 h. A blank run (using 50 ml distilled water instead of sample) was simultaneously conducted with the same procedure after cooling; the solution was transferred to an Erlenmeyer flask. The reflux flask was rinsed thrice, pouring the rinsing water to the Erlenmeyer flask. The solution was diluted to about 300 ml and about 8 drops of phenanthroline ferrous sulphate was added to the solution as an indicator. The solution was titrated against the Mohr's salt and volume required for the color change from blue-green to reddish blue was noted.

The procedure was repeated for the blank, COD was calculated according to the following equation:

$$\textbf{COD} = \frac{8000 \times (Vb_l - Vs) \times M}{\text{Original volume of sample taken mg/l}}$$

Where,
Vb_l = Titer volume for the blank
Vs = Titer volume for the sample
M = Molarity of Mohr's solution

3.5.7. Biochemical oxygen demand (BOD_5)

BOD is a way to assess the amount of oxygen required for aerobic microorganisms to decompose the organic material in a sample of water over a specific time frame. It is the oxygen uptake demand of a source of water. The purpose of this test is to determine the potential of wastewater and other water to deplete the oxygen levels of receiving waters**.**

BOD was determined according to the method No.5210 in Standard Methods for the Examination of Water and Wastewater 20th Edition [133]. BOD is calculated by measuring the dissolved oxygen (DO) of a sample immediately after it is obtained (initial DO) and after a period of five days (final DO). An incubation period of five days will deplete approximately 60-70% of the available material and was chosen as the standard because it would take the microorganisms in a wastewater sample twenty or more days to decompose all the available organic material.

According to Standard Methods 5210B, the working range is equal to the difference between the maximum initial DO (7-9 mg/l) and minimum DO residual of 1mg/l corrected for seed, and multiplied by the dilution factor.

$$BOD_5 \text{ (mg/l)} = \frac{(D_1 - D_2) - (S)\ Vs}{P}$$

Where:
D_1: DO of diluted sample immediately after preparation, mg/l,
D_2: DO of diluted sample after 5 d incubation at 20°C, mg/l,
S : Oxygen uptake of seed = ΔDO/ml seed suspension added per bottle (6d) (S) = 0 if samples are not seeded),
Vs: Volume of seed in the respective test bottle, ml,
P : Decimal volumetric fraction of sample used; 1/P = dilution factor.

3.5.8. Determination of crude protein

The total nitrogen was determined by using Kjeldahl method (A.O.A.C., 2000) [(134)]. The crude protein content was then calculated by multiplying the total nitrogen by a factor of 6.25.

Procedure:

- Sample (0.5-1.0gm) was placed in a Kjeldahl digestion flask; 5 g K_2SO_4 + 0.5 g $CuSO_4$ and 25 ml conc. sulphuric acid were added to the sample.
- The flask was swirled in order to mix the contents thoroughly then placed on heater to start digestion till the mixture become clear (blue green in color). It needs 2 h to complete.
- 20 ml deionized water was added to the sample after allowing it to cool. Then adding 25 ml NaOH (40%), the sample was then distilled and the liberated ammonia was collected in boric acid and titrated with 0.1N hydrochloric acid.
- A blank was prepared and treated in the same manner except that the tube was free of sample. Protein percentage was calculated according to the following equation:

$$\text{Crude protein (\%)} = \frac{\text{(Sample titre – blank titre) x 14 x 6.25 x 100}}{\text{Sample weight}}$$

Where,
14 : Molecular weight of nitrogen
6.25: The nitrogen factor.

3.5.9. Determination of crude fat

Crude fat was estimated by employing solvent extraction using a Soxhlet extraction unit in dry or liquid sample (A.O.A.C., 2000) [(134)].

Procedure

- One gram sample was weighed into an extraction thimble and covered with absorbent cotton. 50 ml solvent (petroleum ether) was added to a pre-weighed cup. Both thimble and cup were attached to the extraction unit.
- The sample was subjected to extraction with solvent (Petroleum ether; boiling fraction 40-60 $^{\circ}$ C or Hexane) for 30 min followed by rinsing for 1.5 h. The solvent was evaporated

from the cup to the condensing column. Extracted fat in the cup was placed in an oven at 110°C for 1 h and after cooling.

The crude fat was calculated using the following equation:

$$\text{Crude fat (\%)} = \left(\frac{\text{Extracted fat}}{\text{Sample weight}}\right) \times 100$$

3.5.10. Determination of total ash

Total ash content was determined as total inorganic matter by incineration of a sample at 600°C (A.O.A.C., 2000) [134].

Procedure

Sample (1g) was weighed into a pre-weighed porcelain crucible and incinerated overnight in a muffle furnace at 600°C. Then cooled in desiccator and weighed. Ash content was calculated according to the following equation:

$$\text{Ash (\%)} = \left(\frac{\text{Ash weight}}{\text{Sample weight}}\right) \times 100$$

3.5.11. Determination of crude fiber

Crude fiber in the samples was determined by the method described by (A.O.A.C., 2000) [134].

Procedure

- Defatted sample (1g) was placed in a glass crucible and attached to the extraction unit, 150 ml boiling 1.25% sulphuric acid solution was added.
- The sample was digested for 30 min and then the acid was drained out and the sample was washed with boiling distilled water. After that, 1.25% sodium hydroxide solution (150 ml) was added.
- The sample was digested for 30 min, the alkali was drained out and the sample was washed with boiling distilled water.
- Finally, the crucible was removed from the extraction unit and dried at 110°C overnight.
- The sample was allowed to cool in a desiccator and weighed (W1). The sample was then ashed at 550°C in a muffle furnace for 2 h, cooled in a desiccator and reweighed (W2). Extracted fiber was expressed as percentage of the original undefatted sample and calculated as following:

$$\text{Crude fiber (\%)} = \frac{\text{Digested sample (W1)} - \text{Ashed sample (W2)}}{\text{Weight of sample}} \times 100$$

3.5.12. Determination of carbohydrate

Carbohydrate content where determined by the difference according to (Smith *et al*., 1956) [135].

3.5.13. Determination of sodium chloride

Sodium chloride was determined according to Mohr's method (Mohr, 1856) [136]. It depends on the titration of NaCl sample with standard AgNO3 using potassium chromate (K_2 Cr O_4) as indicator.

The reactions are summarized as follow:

Before end point: $Ag^+ + Cl^- \rightarrow AgCl$

At end point: $2Ag^+ + CrO_4^{2-} \rightarrow Ag2CrO4$

1-2 g of sample was dissolved in 100 ml of distilled water, 2:3 drops of phenolphthalein indicator was added, nitric acid was used for neutralization (from pink color to colorless), 2ml of potassium chromate indicator was added, then titration by silver nitrate (0.1N) until the color reddish brown at end point.

Calculation:

$$\% \text{ Nacl} = \frac{V \times N \times 5.85}{\text{Weight of sample}}$$

Where:

V= Volume of standard solution of silver nitrate,

N = Normality of standard solution of silver nitrate.

3.6. Precipitation of soluble proteins

Lowering the pH near to the isoelectric point reduces the repulsive forces and allows the proteins to associate. Therefore, many proteins exhibit minimum solubility at the isoelectric point (IEP) where the lack of electrostatic repulsion promotes aggregation between protein molecules. Due to aggregation of proteins at these conditions they can be removed from solution by appropriate centrifugal force.

In order to find the most appropriate pH to solubilize and recover proteins from a protein solution, a solubility curve can be constructed (protein concentration vs. pH). Protein solubility was determined by the method of (Lee *et al.*, 1992) [137] with slight modification to determine the effect of the recovery process on the amount of recovered protein. The recovered proteins were diluted to 1 g/100 ml with distilled water and centrifuged for 20 min at 10,000 x g to sediment insoluble proteins. Protein concentrations in the total solution and supernatant fractions were determined by Lowry method and Protein solubility was computed as:

$$\text{Protein Solubility \%} = \frac{\text{g protein in the supernatant} \times 100}{\text{g total protein}}$$

Sample (150 ml) of water-soluble protein from rice starch manufacture wastewater (protein concentration 0.51g/100 ml) was changing its pH by using 1N sulfuric acid or 1N sodium hydroxide from (3 to 9) at different time and at ambient temperature. Samples were then evaluated on the basis of pH, time related effects on precipitation. The percentage of protein precipitated was assessed by determining the concentration of water- soluble protein in the supernatant.

3.7. Analysis of dried separated material

Ten parameters were investigated for the dried separated material .Some of these parameters were investigated also for the wastewater (as : Crude Protein content, Crude Fat, Total Ash, Crude Fiber, Carbohydrate content; and sodium Chloride) to find their extraction percentage. But the other four parameters are: Moisture content, total phenol content, Amino Acids content; and Aflatoxins content.

3.7.1. Determination of moisture

The moisture content in each sample was determined according to the procedure described in method No 925, 10. A.O.A.C, (2000) [134].

Procedures:

1- Empty dish with lid was dried in the oven at 105 ˚C for 3 h and transfer to desiccator to cool.
2- Empty dish was weighted
3- Three grams from the sample were put in the dish and weighted again.
4- The dish with sample was put in the oven for 3 h at 105 ˚C.
5- After drying, the dish with partially covered lid was taken to the desiccator to cool.
6- The dish was reweighted.

Calculation:

$$\text{Moisture \%} = \frac{(W_1 - W_2)}{W_1} \times 100$$

Where:

W_1 = Weight (g) of sample before drying
W_2 = Weight (g) of sample after drying

3.7.2. Determination of total phenol

The content of total phenolic compounds were measured using the Folin & Ciocalteau method in Central Lab, National Institute of Oceanography and Fisheries, Ministry of State for Scientific Research of Egypt, by the method of Taga *et al*. (1984) [138] the Folin-Ciocalteu method, based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product. The Folin-Ciocalteu reagent, diluted 10 times (2.5 ml) was mixed with 2 ml of saturated sodium carbonate (75 g/L) and 50 µl of sample (supernatant) and homogenized for 10 s and heated for 30 min at 45°C. The absorbance was measured at 765 nm after cooling at room temperature. The data were calculated by comparison between a standard curve (212-1062 µmol gallic acid/L) and the absorbance of each sample. The data were expressed as µmol gallic acid equivalents per gram of dry matter.

3.7.3. Determination of aflatoxins

The aflatoxins are secondary metabolites produced primarily by *Aspergillus flavus* and *Aparasiticus*. Aflatoxins are common crop contaminants, with contamination occurring in the field, during harvest or during storage. The major aflatoxins are aflatoxin B1, B2, G1 and G2.

The aflatoxins, as a group (AFB1, AFB2, AFG1, AFG2 and AFM1), are classified as group 1 carcinogens (International Agency for Research on Cancer, 2002) [139]. The European Union allowable limits for AFB1 in animal feeds and concentrates are 20 and 5mg/kg, respectively (European Community, 2003) [140]. The HPLC analysis in Central Lab, Faculty of Agriculture, Alexandria University, was used for checked AFB1 concentration in animal feed according to the method No 970.44 (A.O.A.C., 1995) [141].

3.8. Nutritional analysis of diets

3.8.1. Determination of dry matter

Dry mater is the residue obtained after a total evaporation of water by heating the sample. It is used to correct the value of chemical analysis in order to express it on the same dry matter basis. Dry matter is estimated according to A.O.A.C. (1998)[142], where 3 g of sample (W1) dried until constant weight at 103°C to 104°C for 3 h, cool immediately after drying in a desiccator and weight soon after reaching room temperature (W2).

Dry matter (%) = (W2/W1) x100.

3.8.2. Determination of organic matter

Organic matter is determined according to A.O.A.C. (1998) [142]

where:

Organic matter % = 100 - Ash%

3.8.3. Determination of nitrogen free extract

Nitrogen free extract is calculated according to A.O.A.C. (1998) [142]

where:

Nitrogen free extract % = 100 – (Water%+ Crude protein% + Crude Fiber% + Ether extract% + Ash %)

3.8.4. Determination of gross energy

The Gross energy calculated according to MAFF (1975) [143]

where:

Gross energy in mega joule per kilogram of dry matter = 0.0226 Crude protein + 0.0407 Ether Extract + 0.0192 Crude Fiber + 0.0177 Neutral detergent fibers (A.O.A.C., 1998) [142]

3.8.5. Determination of digestible energy

The Digestible Energy is calculated according to A.O.A.C. (1998)[142]

where:

Digestible Energy in kilocalorie per kilogram = 4.36 – 0.0491 x Neutral detergent fibers%

3.8.6. Determination of neutral detergent fibers

The Neutral detergent fibers percent is calculated according to A.O.A.C. (1998) [142]

where:

Neutral Detergent Fiber % = 2.92 + 0.657 X Crude Fiber%

3.9. Biological experiment

3.9.1 Experimental animals

Forty male albino rats, 2 months old, with average body weight of 200±50g were obtained from National Research Institute, Cairo, were used to study the effect of using new rice starch manufacture by-product as animal feed on some hematological, biochemical parameters of blood serum and attributed with some histopathological studies in kidney and liver. Rats acclimated for one week prior to the experiment and housed in Universal galvanized wire cages.

3.9.2. Experimental design

The animals were randomly divided into 4 groups, each of 10 rats. Group one served as a control (C) and fed normal diet while group T_1 to T_3 served as the treated groups. Group T_1 fed 95% control diet + 5% new rice starch manufacture by-product, group T_2 fed 90% control diet +10% new rice starch manufacture by-product, group T_3 fed 80% control diet+20% new rice starch manufacture by-product. The feeding started at 18 January 2014 and lasted for a period of eight weeks under local weather conditions for the city of Alexandria.

At the end of the treatment period, animals were slaughtered and blood samples were obtained from the Aorta of each animal, 1ml were collected on heparinized tubes for the hematological studies, while the rest were collected in test tubes, placed immediately on ice. Serum was obtained by blood centrifugation at 3,000 xg for 20 min. and stored at -20 ºC until analyzed. The rats were weighted before slaughter.

Selected internal organs were extraction and weighted samples from the liver and kidneys were taken from the different groups and preserved in 10% formalin for histological examination.

3.9.3. Diet preparation

In Nubaria Station, Animals Production Research Institute, Agriculture Research Center, Ministry of Agriculture, basic experimental diet pellets used as diet for control rats during the experimental period were prepared in feed mixing unit consisting of 36% wheat bran, 15% soybean meal, 40% yellow corn, 6% molasses, 1% limestone, 1% common salt and 0.5% vitamins minerals premix as in Table (19), rice starch manufacture by-product (RSMBP) were mixed at ratios 5%, 10% and 20% to basic control diets required to feed the treatment groups of rats during the experimental period .

3.9.4. Data collected

Total body weight of the animals was recorded at the beginning and during the experiment period. The rats had free access to diets and tap water. The actual weight of food intake and feed efficiency were calculated. Rats were weighed weekly to determined body weight gain.

3.9.5. Growth performance parameters

Body weight was recorded biweekly prior to access to food at 9 am, feed intake and water intake were recorded daily and calculated as weekly mean, weight gain (WG) and Feed efficiency ratio (FER) were calculated according to Marai *et al*. (2007)[144] as follow:

Weight gain (WG) = final body weight – initial body weight
Feed efficiency ratio (FER) = mean food consumed / mean weight gained

Table (19): Composition of basic experimental diet

Component	Amount	
Wheat bran	36	%
Soybean meal	15	%
Yellow corn	40	%
Molasses	6	%
Limestone	1.5	%
Salt (Na Cl)	1	%
Vit. and Min.	0.5	%
Total	100	%
Vitamins and minerals		
Vitamin A	$12x10^6$	IU/ton
Vitamin D3	$3 x10^6$	IU/ton
Vitamin E	10	gm/ton
Vitamin K	2	gm/ton
Vitamin D1	1	gm/ton
Vitamin D3	6	gm/ton
Vitamin D6	1.5	gm/ton
Vitamin D12	12	gm/ton
Choline	260	gm/ton
Pantothenic acid	10	gm/ton
Nicotinic acid	30	gm/ton
Folic acid	1	gm/ton
Biotin	50	mg/ton
Manganese	70	gm/ton
Zinc	50	gm/ton
Ferrous	30	gm/ton
Copper	8	gm/ton
Iodine	0.3	gm/ton
Selenium	0.1	gm/ton
Cobalt	0.1	gm/ton

According to N.R.C. (1995) [145]

3.9.6. Blood analysis

At the end of the experiment, blood samples were obtained from each animal, 1ml were collected on heparin for the hematological studies, while the rest were collected in test tubes, placed immediately on ice. Serum was obtained by blood centrifugation at 3,000 x g for 20 min. and stored at -20 ºC until analyzed.

3.9.7. Hematological analysis

Whole heparinised blood was analysed shortly after collection for hemoglobin (Hb), blood hematocrit (HCT), red blood cell (RBC) counts and white blood cell (WBC) counts.

Hemoglobin concentration was determined in whole blood shortly after collection using Hb kits obtained from Diamond Diagnostics Egypt. RBC's were counted on an American Optics **(AO)** bright line hemocytometer using a light microscope at 430 X magnification. Blood samples were diluted 200 times with physiological saline (0.9 % sodium chloride solution) before counting. Microhematocrit tubes with a hematocrit centrifuge (5 min. at 16,500 X g) were used to determine HCT. WBC's were counted on an **AO** bright line hemocytometer using a light microscope at 100 X magnification after diluting blood samples 20 times with a diluting fluid (1% acetic acid and little of Leshman's stain) before counting.

Wintrobe indices were calculated according to Greer *et al.* (2003) [146] as follow to determine the changes in red blood cells:

MCV (cu μ) = HCT (%) / No. of RBC's (per 100ml)
MCH (pg) = Hb (g per 100ml) / No. of RBC's (per 100ml)
MCHC (%) = [Hb (g per 100ml)/ HCT (%)] X 100

3.9.8. Blood serum analysis

3.9.8.1. Determination of blood serum glucose

Glucose was determined according to Emerson (1977) [147]. The principal of this method is according to the following reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4- amino- antipyrine} \xrightarrow{\text{peroxidase}} \text{Red quinonciminc} + 4H_2O$$

100 mg/dl of glucose was used as standard, 10 μl of standard and serum samples were added to 1.0 ml of working solution which is composed of 150 mmol/l phosphate buffer, 10 mmol/l phenol 10,000 IU/l glucose oxidase, 300 IU/l peroxidase and 4-aminoantipyrine, mix and incubate for 10 min 37°C water bath to allow the development of color. Absorbance was measured against the control solution using 1 cm thick cell at wavelength 505 ηm.

3.9.8.2 Determination of blood Serum total protein

Serum total protein (TP) was measured by the Biuret method as described by Armstrong and Carr (1964) [148]. The principal of this method is that protein forms violet complex with cupric ions in alkaline media; analytical curve was plotted using crystalline

albumin as standard. Biuret reagent is composed of 173 g sodium citrate and 100 g of sodium carbonate are dissolved in 300 ml of distilled water by heating the mixture in a water bath. 17.3 g of $CuSO_4$ is dissolved in another 300 ml of distilled water. Both solutions were poured into a 1000 ml graduated flask and made up to the mark with distilled water, mix the contents with shaking. 0.1 ml of solution from each of the four standard test tubes containing standard albumin solution was transfered, and the samples; add 5 ml of Biuret reagent. Leave the solution for 30 min for color development. Absorbance was measured against the control solution using 1 cm thick cell at wavelength 550 ηm.

3.9.8.3 Determination of blood serum albumin

Albumin concentration was determined according to Doumas *et al*. (1971) [(149)]. The principal of this method is that when albumin is added in a buffered solution of bromocresol green dye, a blue color appears and is proportional to the albumin concentration in the sample and is measured photometrically. Crystalline bovine albumin 5% w/v was used as standard, 10 g / l of standard and samples were added to 1.5 ml of working reagent which is composed of 0.1047 g / l bromocresol green, 11.2185 g /l succinate buffer pH 3.8 and 9 g of Tween 20 were dissolved till 1000 ml of distilled water in measuring flask, the contents were mixed with shaking. The solutions were left for 1 min for color development. absorbance was measured against the control solution using 1 cm thick cell at wavelength 628 ηm.

3.9.8.4 Determination of blood serum total lipids

Total lipids (TL) were determined as described by Frings *et al*. (1972)[(150)], the principal of this method is that lipids are hydrolysed by sulphuric acid, then treated with phosphovanilline mixture to produce sulpho-phosphovanilline complex of rose coloration which is measured photometrically. Total lipids (8 g /l) was used as standard, 100 µl of standard and samples were added to 3 ml of concentrated sulphuric acid, mixed well and let to boil in boiling water bath for 10 min, then cooled, the hydrolysates were homogenized, 100 ml of the hydrolysate were added to the working reagent consisted of 8 mmol/l vaniline is dissolved in 13.9 mol/l phosphoric acid, the contents mixed with shaking. The solutions were set at 37 °C for 10 min in water bath to allow color development. The absorbance was readed against the control solution using 1 cm thick cell at wavelength 525 ηm.

3.9.8.5 Determination of blood serum cholesterol

Total cholesterol concentration was measured according to Watson (1960)[(151)]. The principal of this method is that cholesterol reacts with acetic anhydride and concentrated sulphuric acid yielding a blue-green color, which is measured photometrically. 200mg/dl of cholesterol was used as standard, 100 µl of standard and samples were added to 2.5 ml of working reagent which is composed of 620 ml acetic anhydride dissolved in 380 ml glacial acetic acid, mix the contents with shaking. The solutions were set in cold water bath; 0.5 ml concentrated sulphuric acid was add to allow color development. Absorbance was read against the control solution using 1 cm thick cell at wavelength 550 ηm.

3.9.8.6 Determination of blood serum HDL cholesterol

High density lipoprotein cholesterol (HDL) concentration was measured according to Richmond (1973) [152]. The principal of this method is that low and very low density lipoprotein cholesterol is precipitated quantitatively by the addition of phosphotungestic acid in the presence of magnesium ions. After centrifugation the cholesterol concentration in the HDL fraction, which remains in the supernatant, is determined as the residues of cholesterol photometrically. 500 µl of standard and samples were added to 50 µl of precipitate reagent, 13.9 mmol/l phosphotungastic acid and magnesium chloride 570 mmol/l, mixed and allowed for 10 min at room temperature; then centrifuged at 1,320 x g for 10 min, then determine the residue cholesterol in the supernatant using the cholesterol procedure. Absorbance was read against the control solution using 1 cm thick cell at wavelength 550 ηm. Calculate the HDL concentration by the following equation:

HDL (mg/dl) =A sample X 180
where: A = absorbance, 180 = extinction value

3.9.8.7. Determination of blood serum LDL cholesterol

Low density lipoprotein cholesterol (LDL) concentration was measured according to Assmann *et al*. (1984) [153]. The principal of this method is that low density lipoprotein cholesterol is precipitated by the addition of heparin at pH 5.4. After centrifugation the HDL and VLDL fractions remain in the supernatant, which can then be determined as the residues of cholesterol photometrically,

100 µl of standard and samples were added to 1000 µl of precipitate reagent which is composed of heparin 50,000 IU/l and sodium citrate 0.064 mol/l pH 5.04, mix and allow for 10 min at room temperature; the mixture was centrifuged at 1320 x g for 10 min, then determine the residue cholesterol in the supernatant using the cholesterol procedure. Read the absorbance against the control solution using 1 cm thick cell. Calculate the LDL concentration by the following equation:

LDL = Total cholesterol – Cholesterol in the supernatant

3.9.8.8. Determination of blood serum triglycerides (TG)

Triglycerides (TG) concentration was measured according to Bucolo and David (1973)[154]. The principal of this method is that triglycerides are involved in a series of reactions as follows:
Triglycerides are hemolyzed by lipoprotein lipase (LPL) to glycerol

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol + Fatty acids}$$

Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK)

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate + ADP}$$

The oxidation of glycerol-3-phosphate is canalized by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2)

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

In the presence of peroxidase (POD), hydrogen peroxide affects the oxidative cuppling of 4-chlorophenole and 4-aminoantipyrine (4-AAP) to form a red color quinoneimine dye which is measured photometrically.

$$2\ H_2O_2 + \text{4-AAP} + \text{4-chlorophenole} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\ H_2O$$

Triglycerides 200 mg/dl was used as standard, 10 µl of standard and samples were added to 1.0 ml of working reagent which is composed of 50 mmol/l pipes buffer pH 7.0, 6 mmol/l 4-chlorophenol, 0.5 mmol/l magnesium aspartate, 10000 IU/l lipase, 2.0 KIU/l peroxidase, 1.0 mmol/l 4-aminoantipyrine, 3500 IU/l glycerol-3-phospate oxidase, 750 IU/l glycerol kinase, 1.0 mmol/l ATP and 8 mmol/l sodium azide; the miture was mixed and incubated for 5 min at 37 °C in water bath to allow the reaction develop. Absorbance was read against the control solution using 1 cm thick cell at wavelength 546 ηm.

3.9.8.9. Enzymes activity

3.9.8.9.1. Determination of blood serum aspartate aminotransaminase (AST)

Aspartate aminotransaminase (AST) was measured by the method of Reitman and Frankel (1957)[(155)]. The principal of this method is that aspartate amino transaminase catalyzes the following reaction:

$$\text{L- aspartate} + \alpha\text{-oxoglutarate} \longrightarrow \text{L- glutamate} + \text{oxaloacitate}$$

The oxaloacitate which is formed is assayed by the formation of 2,4-dinitrophenylhydrazine which yields a reddish-brown color in alkaline medium and is measured photometrically, incubate 0.5 ml of AST for 5 min at 37 °C, AST substerate which is composed of 0.1 mol phosphate buffer pH 7.4, 0.002 mol α-oxoglutarate and 0.2 mol L-aspartic acid, add 0.1 ml of blank (distilled water) or samples to the reagents, then mixed and incubated at 37 °C in water bath for 30 min. Add coloring reagent, 2-4 dinitrophenyl-hydrazine, then left for 20 min at room temperature, add the alkaline reagent, 0.4 N NaOH then let for 5 min. Absorbance was readed against the blank solution using 1 cm thick cell at wavelength 546 ηm.

3.9.8.9.2. Determination of blood serum alanine aminotransaminase (ALT)

Alanine aminotransaminase (ALT) was measured by the method of Reitman and Frankel (1957) [(155)]. The principal of this method is that alanine aminotransaminase catalyzes the following reaction:

$$\text{dL- alanine} + \alpha\text{-oxoglutarate} \longrightarrow \text{L- glutamate} + \text{pyruvate}$$

The pyruvate which is formed is assayed by the formation of 2,4-dinitrophenylhydrazine which yields a reddish-brown color in alkaline medium and is measured photometrically, 0.5 ml of ALT substerate was incubated for 5 min at 37 °C, ALT substrate is composed of 0.1 mol phosphate buffer pH 7.4, 0.002 mol α-oxoglutarate and 0.2 mol dL- alanine, 0.1m of blank (distilled water) and samples were added to the reagents, mixed and incubated at 37 °C in

water bath for 30 min., 2-4 dinitrophenyl-hydrazine coloring reagent was added and let stand for 20 min at room temperature, alkaline reagent of 0.4N NaOH was added then let stand for 5 min. Absorbance was readed against the blank solution using 1 cm thick cell at wavelength 546 ηm.

3.9.8.9.3. Determination of blood serum alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) was determined according to Bauer (1982) [156] The principal of this method is that:

$$\text{P-nitrophenilephosphate} + H_2O \xrightarrow{\text{ALP}} \text{phosphate} + \text{p-nitrophenol}$$

The p-nitrophenol which is formed is measured photometrically. Add 1.0 ml of working reagent, which is composed of 0.9 mol/l 2-amino-2-methyl-1-propanol buffer pH 10.5, and 1 mmol/l magnesium sulphate, and 10 ml of buffering reagent, to the substrate reagent consisted of 5.5 mmol p-nitrophenyl-phosphate, then the mixture was incubated at 37 °C exactly for 30 min, alkaline reagent which is composed of 10 ml 0.4N NaOH was added. Absorbance was readed against the blank solution using 1 cm thick cell at wavelength 405 ηm.

3.9.8.9.4. Determination of blood serum acid phosphatase (ACP)

Acid phosphatase (ACP) was determined according to Andersch and Szecyphinski (1947) [157]. The principal of this method is that:

$$\text{o-nitrophenylephosphate} + H_2O \xrightarrow{\text{ACP}} \text{phosphate} + \text{p-nitrophenol}$$

p-nitrophenol which is formed is measured photometrically. 0.2 ml of samples was added to 1.0 ml of working reagent which is composed of 10 ml of citrate pH 4.8, 5.5 mmol p-nitrophenyl-phosphate, incubate at 37 °C exactly for 30 min, Alkaline reagent was added, 10 ml 0.02N NaOH. Read the absorbance against the blank solution using 1 cm thick cell at wavelength 405 ηm.

3.9.8.9.5. Determination of blood serum α-amylase

α-amylase was determined according to Stroev and Makarova (1989)[158]. The principal of this method based on photometric determination the differance of starch consumption during starch hydrolysis reaction by α-amylase of blood serum. 0.5ml of 2 g/dl starch solution was added to 0.3 ml of 0.1 M phosphate buffer pH 7.2 and 0.1 ml 3 g/l of sodium chloride solution, and incubated at 37°C for 10 min to the sample solution 0.1 ml of blood serum was added, and to standard solution 0.1 ml of 1M HCl was added, all tubes were shaken thoroughly and placed for incubation at 37°C for 30 min, the reaction was terminated by adding 0.1 ml of 1M HCl. 0.2 of the mixtures were then added to 4.3 ml distilled water and 0.5 ml potassium iodide solution (0.1 M iodine and 3 g/dl potassium iodide). Absorbance was read immediately against the blank solution using 1 cm thick cell at wavelength 650 ηm. calculation was carried out by the equation:

$$X=[(E_{\text{control}} - E_{\text{sample}})\ 0.01 \times 2 \times 1000]/\ (E_{\text{control}})\ 0.1$$

3.9.8.10. Determination of blood serum urea

Urea was determined using commercially available kits obtained from Bio ADWIC; Egypt according to Patton and Crouch (1977)[159], the principal of this method is according to the following react

$$\text{Urea} + H_2O \xrightarrow[\text{Urease}]{} 2NH_3 + CO_2$$

In an alkaline medium, the ammonium ions react with salicylate and hypochloride to form a green colored indophenol which is measured photometrically; urea 50 mg/dl was used as standard, 10 µl of standard and samples were added to 100 µl of buffering reagent (1). It is composed of 100 mmol/l phosphate buffer pH 8, 52 mmol/l sodium salicilate, 2.9 mmol/l sodium nitroprusside and 2 mmol/l EDTA are dissolved till 1000 ml of distilled water in measuring flask, add one drop of reagent (2) (enzyme reagent). >5000 IU/l urease enzyme, mix and incubate for 3 min at 37 °C water bath to allow the reaction develop, add reagent (3) (alkaline reagent) 80 mmol/l sodium hydroxide and 4 mmol/l sodium hypochlorite, solution was mixed and incubated for 5 min at 37 °C water bath to allow color developed. Absorbance was readed against the control solution using 1 cm thick cell at wavelength 580 ηm.

3.9.8.11. Determination of blood serum creatinine

Creatinine was determined using commercially available kits obtained from Bio ADWIC, Egypt according to Larsen (1972) [160]. The principal of this method is that creatinine in alkaline solution reacts with picrate yielding a yellow color complex which is measured photometrically .Creatinine 2 mg/dl was used as standard, 500 µl of standard and samples were added to 1.0 ml of working reagent. Working reagent is formed fresh before using from equal volumes of reagent (1). It is composed of 38 mmol/l of picric acid. And reagent (2) which is composed of 1.2 mmol/l sodium hydroxide, mix the contents with shaking. Absorbance was read against the control solution, after 30 sec. and 120 sec using 1 cm thick cell at wavelength 546 ηm, calculate the kinetic difference between the initial and final absorbance to determine the creatinine concentration.

3.9.8.12. Determination of blood serum total bilirubin

Total bilirubin was determined according to Jendrassik and Grof (1938) [161]. The principal of this method is that: Bilirubin is cupped with diazotized sulphanilic acid in the presence of caffeine to give an azo dye. Normal saline is used instead of caffeine when direct bilirubin is determined photometrically, For total bilirubin, 0.2 ml of reagent (1) was added, which is composed of 31 mmol/l suphanilic acid and 0.2 N HCl, to one drop of reagent (2), which is composed of 28 mmol/l sodium nitrite, and 1.0 ml of reagent (3), which is composed of 0.28 mol/L caffeine and 0.55 mol/l sodium benzoate. Then to the all reagents 0.2 ml samples were added. Blank was made by adding reagents (1) and (3) to sample; mix and allow for 10 min at room temperature, add 1 ml of reagent (4), which is composed of 0.99 mol/l tartarate and 2.0 N of NaOH, solutions were mixed and incubated for 5 min at room temperature. Absorbance was read against the blank solution using 1 cm thick cell at wavelength 578 ηm.

3.9.8.13. Determination of blood serum uric acid

Uric acid was determined according to Barham and Trinder (1972)[162] . The principal of this method is according to the following reaction:

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{3.5, Dichloro-2-hydroxybenzensulphonate} +$$

$$\text{4-Amino antipyrine} \xrightarrow{\text{Peroxidase}} H_2O + HCl + \text{Colored quinoneimine}$$

6 mg/dl of uric acid was used as standard, 5 µl of standard and serum samples were added to 1.0 ml of working solution which is composed of 50 mmol/l tris buffer, 5 mmol/l 3.5, dichloro-2-hydroxybenzensulphonate, 500 IU/l uricase and 2000 IU/l peroxidase and 0.20 mmol/l 4aminoantipyrine, mix and incubate for 10 min at 37°C water bath to allow the development of color. Absorbance was measured against the control solution using 1 cm thick cell at wavelength 510 ηm.

3.9.8.14. Determination of thiobarbituric acid–reactive substances (TBARS)

Thiobarbituric acid–reactive substances (TBARS) were measured as described by Tapel and Zalkin (1959) [163]. 1 ml of serum was added to 2 ml of 7.5% trichloroacetic acid and mixed. The mixture was centrifuged at 1000 xg for 10 min. 2 ml of supernatant added to 1 ml of 0.7 % 2- thiobarbituric acid. After boiling for 10 min, the reactants were cooled and TBARs were measured at 532 nm. An extinction coefficient of 156.000 $mol^{-1}cm^{-1}$ was used for calculation.

3.10. Histopathological studies

Parts of kidney and liver tissues were fixed in 10 % formaldehyde solution, embedded in paraffin wax, and cut with microtome for 5µ thick sections. The sections were stained by Hematoxylin and Eosin (H&E) stains and microscopically studied to evaluate its morphology.

3.11. Statistical analysis

Statistical analysis in water and wastewater analysis was carried out on the entire data collected using the average values as follow:

The sum of all the values divided by their number, or

$$\bar{x} = \frac{\Sigma x}{n}$$

Statistical analysis in biological evaluation was carried out on the entire data collected using one-way ANOVA and Duncan New Multiple Range test from SPSS 16.0 package to compare the mean values of the treated groups with the control (SPSS, 2006)[164].