

FERMENTATIVE TECHNOLOGICAL RESEARCH FOR OBTAINING ZINC ENRICHED YEASTS

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ABSTRACT: The influence of inorganic zinc solution at different time of cultivation on zinc accumulated in yeast biomass was tested. It has been noticed that there are yeasts which ferment glucose and sucrose as carbon source. A yeast (wine and beer) consortium can be the solution for fermentation of glucose and sucrose in order to develop a high content in biomass weight. It has been observed that the total zinc in yeast biomass obtained at pilot level was 1100-1760 mg/kg and the organic zinc 420-720 mg/kg. For control samples (spent brewing yeast) was found 68 mg/kg yeast total Zn and 48-68 mg/kg yeast organic zinc.
The wet yeast biomass enriched with zinc was used to be mixed with wet spent brewing yeast biomass. After the mixture was obtained, the samples were dried using a drum dryer and were analyzed for the content in total and organic zinc. For mixture, the content of total zinc was 90 mg/kg yeast and 70-90 mg/kg/yeast organic zinc
KEYWORDS: brewing yeast, total zinc, organic zinc, biomass, feed

1. INTRODUCTION (HEADING 1)

Zinc has a wide array of vital physiological functions. It has a catalytic role in each of the six classes of enzymes [1]. The majority of dietary zinc is absorbed in the upper small intestine. It is known that zinc in inorganic form may not be as bioavailable as organically bound zinc. Silvia Šillerová [2] investigate the accumulation in yeast cells of three inorganic zinc salts. The highest amount of zinc in yeast cells (18.5 $mg.g^{-1}$) was obtained when added in the form of zinc nitrate in concentration of 200 $mg.100\ mL^{-1}$ of YPD medium. Zinc accumulation and the growth of *Saccharomyces cerevisiae* were investigated by Somayeh Kamran Azad [3] in a culture with zinc sulfate-supplemented medium. It was observed that in the presence of 30 $mg\ l^{-1}$ $ZnSO_4$, the Zn content in the biomass increased by 24-fold, to 4132.34 $mg\ g^{-1}$ in comparison to 171.9 $mg\ g^{-1}$ achieved in the basal medium. A few forms of zinc (inorganic and organic) are authorised by EFSA. Methionine-zinc, technically pure, was authorised without a time limit in accordance with Council Directive 82/471/EEC of 30 June 1982 concerning certain products used in animal nutrition [5] as an amino acid for use on ruminants by Commission Directive 88/485/EEC [6]. Zinc chelate of methionine (1:2) (additive) with chemical formula: $C_{10}H_{20}N_2O_4S_2Zn$ is characterised as a powder with a minimum content of 78 % DL-methionine and a zinc content between 17.5% and 18.5% [18] Zinc chelate of amino acids hydrate is recognised as an efficacious source of zinc in meeting animal requirements. The diet supplemented with zinc chelate of amino acids hydrate during 13 weeks resulted in an average of 3% higher zinc concentration in egg yolk as compared with those from hens fed with zinc sulphate [19]. Zinc-enriched yeasts are derived from cultures of specified strains of *Saccharomyces cerevisiae* grown in the presence of zinc chloride or zinc sulphate [20]. Availa®4 is a nutritional feed ingredient for animals that contains a combination of organic zinc, manganese, copper and cobalt (Zinc-5.15%) [9]. Zinc (Zn) is one of the components of the carbonic anhydrase enzyme, which is very

important for providing carbonate ions during eggshell formation. Inhibition of this enzyme goes to decreased bicarbonate ion secretion and consequently greatly reduces eggshell weight [10].
Based on the studies of [11], the batch with the highest Mn supplementation (200 mg/kg feed) and Zn supplementation (160 mg / kg feed) recorded the highest eggshell weight and the highest eggshell breaking strength. The dietary supplementation of organic (chelated with methionine) Mn, Zn, Cu and Cr at high levels (80, 60, 5 and 0.15 mg/kg, respectively) increased the hatchability of the fertilised eggs and hatchability compared to that of the other groups ($P < 0.05$) [13].
The interactions between zinc forms and dosages had a significant effect on egg weight, feed intake, feed conversion ratio, eggshell thickness, shear force and shear stress of bone, and tibia calcium concentration. The highest egg weight and the lowest eggshell thickness were observed for the group fed with nano Zn-oxide at 100 mg/kg in the diet [14]. Zinc chelate of amino acids hydrate is a safe source of zinc for all animal species, considering the maximum authorised contents for total zinc in feedingstuffs [19].
The additive 'Zinc chelate of methionine sulfate' is zinc chelated with methionine in a molar ratio 1:1. with a minimum zinc content of 19.1%. Based on literature studies and a specific study conducted with the additive under assessment, zinc chelate of methionine sulfate is an available source of zinc for all animal species [22].
In 2000 the Scientific Committee on Animal Nutrition (SCAN) was requested by the EC to deliver an opinion on the total maximum authorised zinc content in feed; this value was at that time 250 mg Zn/kg complete feed for all animal species. The SCAN recommended a reduction to 150 mg Zn/kg complete feed for all animal species except piglets (175 mg zinc in case 175 mg Cu/kg feed would be retained) [23]. Regulation (EC) No 1334/2003 sets the following maximum total zinc contents in feed: 250 mg Zn/kg for pet animals; 200 mg Zn/kg for fish and milk replacer and 150 mg Zn/kg for other animal species.
Zinc in the form of its divalent metal ion, Zn^{2+}, is nutritionally essential for all living organisms [15].
Zinc concentrations in plants are in the range 10−200 mg/kg, with cereals, legumes and oilseeds in the range 15−30 mg/kg, oilseed meals between 30 and 125 mg/kg and germ meals (maize, wheat) and beet leaves between 130 and 190 mg/kg [21]. The most commonly used sources of zinc to supplement diets are the oxide (ZnO) and the feed-grade sulphate heptahydrate ($ZnSO_4 \times 7H_2O$). Bioavailability of these feed-grade sources relative to zinc sulphate (analytical-grade) is variable. The present paper related a first stage for obtaining yeast enriched with zinc.

2. MATERIALS AND METHODS

More than 30 colonies of wine and brewing yeast were isolated.
Most of them have been tested for the influence of the carbon source used both for the development of the inoculum and for the fermentation medium over the biomass development.
The maintenance culture
Cultivation condition
Obtaining "stock" maintenance culture (static culture)
The culture media for obtaining the "stock" maintenance culture (wine and brewing selected yeast) are sterilized by autoclaving at 110^0C.
The cultures (wine and brewing selected yeast) were grown on agar tubes (slant) and plates containing volumes of 15 ml cultivation media based on yeast extract, peptone hy-soy, sucrose, agar-agar (YSP–agarised) respectively, at 28 – 30°C for 48h.
Obtaining preinoculum: static culture-wine and brewing selected yeast is obtained from a maintenance culture that is harvested on media based on yeast extract, peptone hy-soy, glucose, agar-agar YPG-agar tubes and incubated at 28-30°C for 34-48 hours.
Culture media for pre-inoculum are sterilized by autoclaving at 120°C.
The preinoculum culture is analyzed for purity and growth rate of the microorganism (wine and brewing selected yeast). The well-developed pure culture is used to prepare the liquid inoculum.
Obtaining the inoculum culture (liquid culture):

- ✓ Culture media used to prepare YPG inoculum: yeast extract 0.5 g%, peptone 0.4 g%, glucose 7 g% from Sigma Aldrich;
- ✓ Culture media used to prepare YSP inoculum: yeast extract 0.5 g%, peptone 0.4 g%, sucrose 7 g% from Sigma Aldrich.

Cultivation conditions: Temperature: 28-30^0C, stirring rate: 140-200 rpm.
The inoculum was prepared from a pre-inoculum culture (stock culture-(wine and brewing selected yeast) in 500 ml Erlenmeyer flasks containing 150 ml YSP/YPG liquid medium. Initial pH of inoculum was 5.0-6.0. The inoculum was incubated for 17-20 hours at 30°C, at 240 rpm, in a shaking incubator.
Stock solutions:
-zinc sulfate solution - prepared by dissolving 6g zinc sulphate heptahidrate (Sigma Aldrich) in 100 ml distilled water, pH ~5.3-5.5

-the solution was filtered using Nalgene syringe filter units, pore size 0.2 µm, 13 mm diameter.

Fermentation: Fermentation has been carried out in a bioreactor, at pH 5.2-5.8 and 30^oC.
The optimization process (first stage) is presented below:
The fermentation process was optimised by variation of the inoculation ratio with the liquid culture wine and brewing yeast -inoculum developed previously (15, 25 and 35%) (table no.1) and the time of addition of inorganic zinc solution during fermentation process. The inoculum was developed from new isolated yeast (brewing and wine yeast) and the process was monitored online and offline.
The zinc was added during the fermentation process in three portions of 100 ml/portion at different stages of development of the yeast cell culture.
Feed yeast is composed of 100% from the brewing yeast (*Saccharomyces cerevisiae*) biomass resulted from the technological process of brewing (spent brewing yeast), which is dried and inactivated with a specific technology. It is presented in the form of a brownish-brown powder with a bitter-sweet taste, having the specific smell and aroma of yeast.

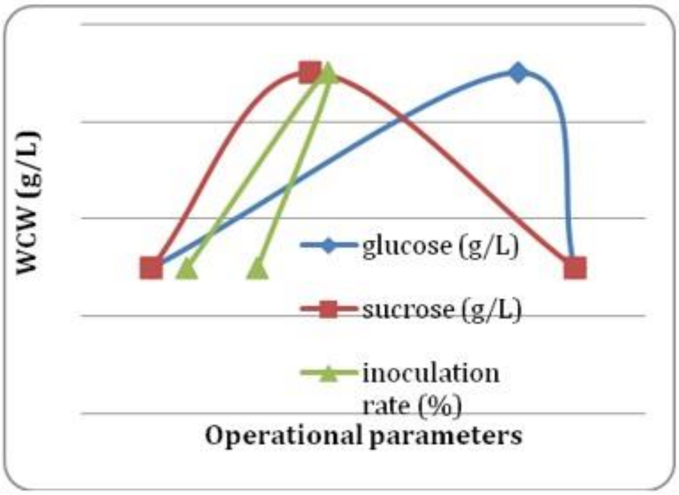

Figure 1. Influence of inoculation rate towards biomass growth

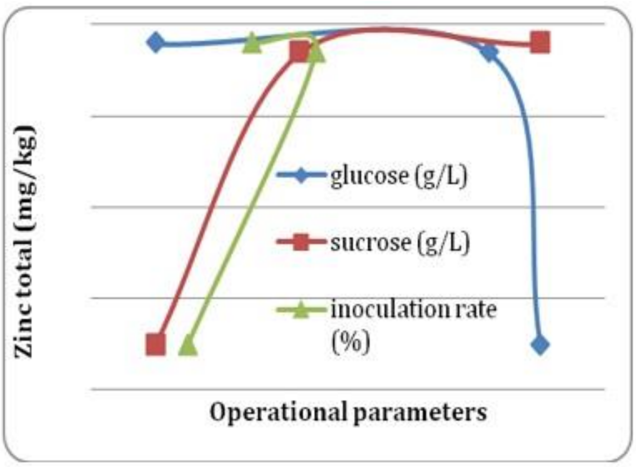

Figure 2. Influence of rate of inoculation towards total zinc incorporated in yeast biomass

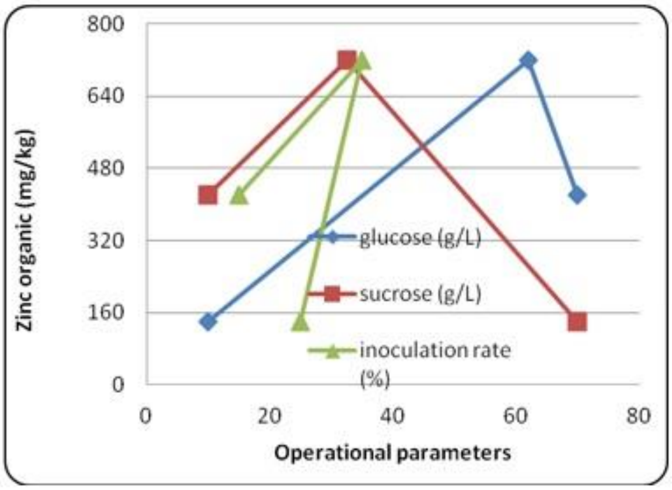

Figure 3. Influence of rate of inoculation towards organic zinc incorporated in yeast biomass

After the fermentation is finalized, the culture fermented media was a subject of the post-fermentation process comprising the following steps:

- ✓ Separation of biomass by centrifugation;
- ✓ Washing the biomass;
- ✓ Drying the biomass.

Table 1. Influence of inoculation ration towards wet cell weight

Batch	Inoculation rate (%)	Wet cell weigh (WCW) (g/l)
S1	25	27
S2	35	31
S3	15	27

Zinc analyses for yeast biomass
According to the protocol:

- first, the total zinc content used after digestion ICP-OES method (inductively coupled plasma optical emission spectrometry method) is being measured;
- second, after the ultrasonic treatment, the inorganic zinc content of samples is measured using SEC-ICP-MS method;
- the organic zinc content of samples is calculated using this equation:

"organic zinc content" =
"total zinc content" - "inorganic zinc content".

Measurement of organic zinc

- ✓ **Size exclusion chromatography (SEC) – inductively coupled plasma mass spectrometry (ICP-MS)**Column: Superdex Peptide 10/300 GL, 10 × 310 mm
- ✓ Mobile phase: 0.5M ammonium-acetate (pH = 5.5)
- ✓ Flow rate 0.9 mL/min

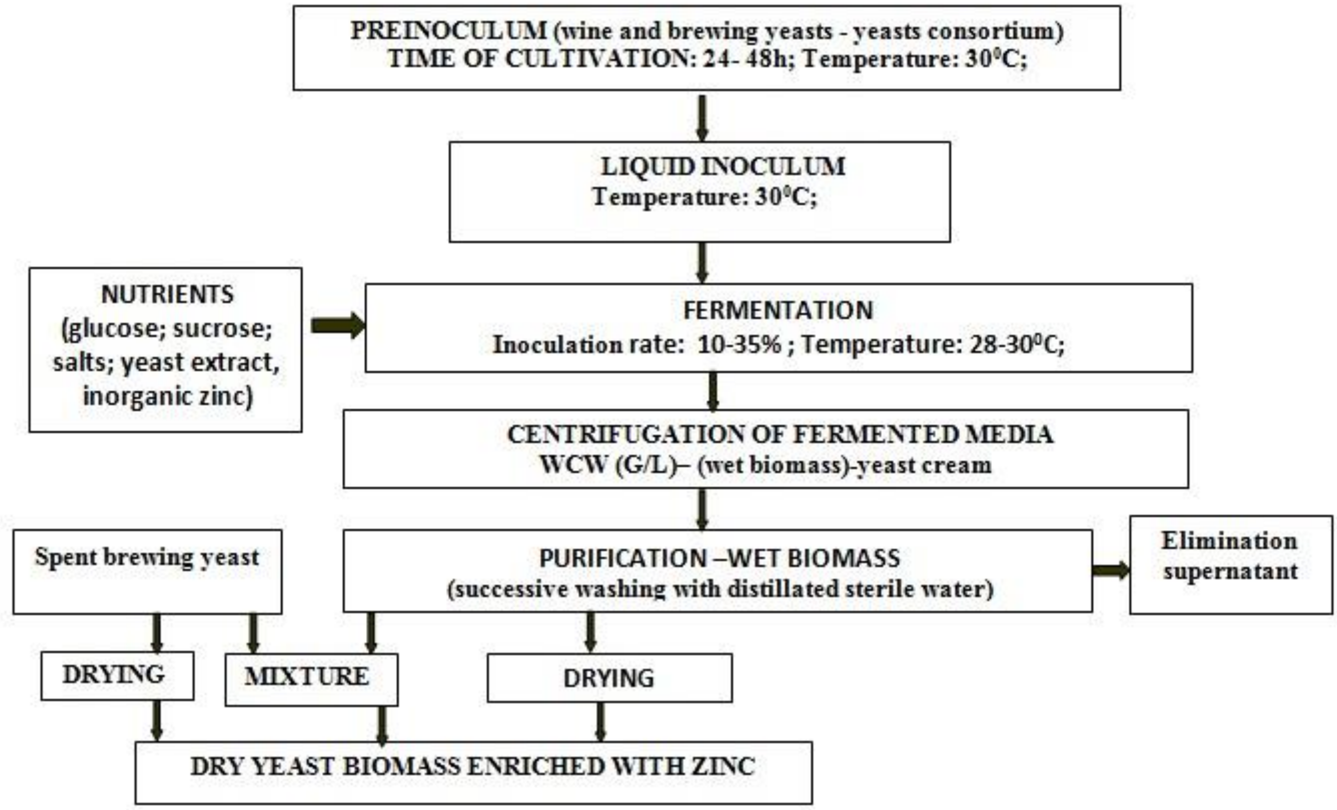

Figure 4. Biotechnological flux for obtaining yeast enriched with zinc

Agilent 7500 ce ICP-MS

- ✓ Time resolved analysis (full quant)
- ✓ No gas mode
- ✓ 66Zn isotope
- ✓ No interference equation
- ✓ Carrier gas 0.95 L/min
- ✓ Makeup gas 0.25 L/min
- ✓ Spray chamber temp: 2 deg
- ✓ Plasma forward power: 1600 W
- ✓ V-groove nebuliser.

Procedure

- 0.1 g lyophylised sample in 15 mL PP centrifuge tube
- 6 mL H_2O
- **Ultrasonic treatment 2 min**

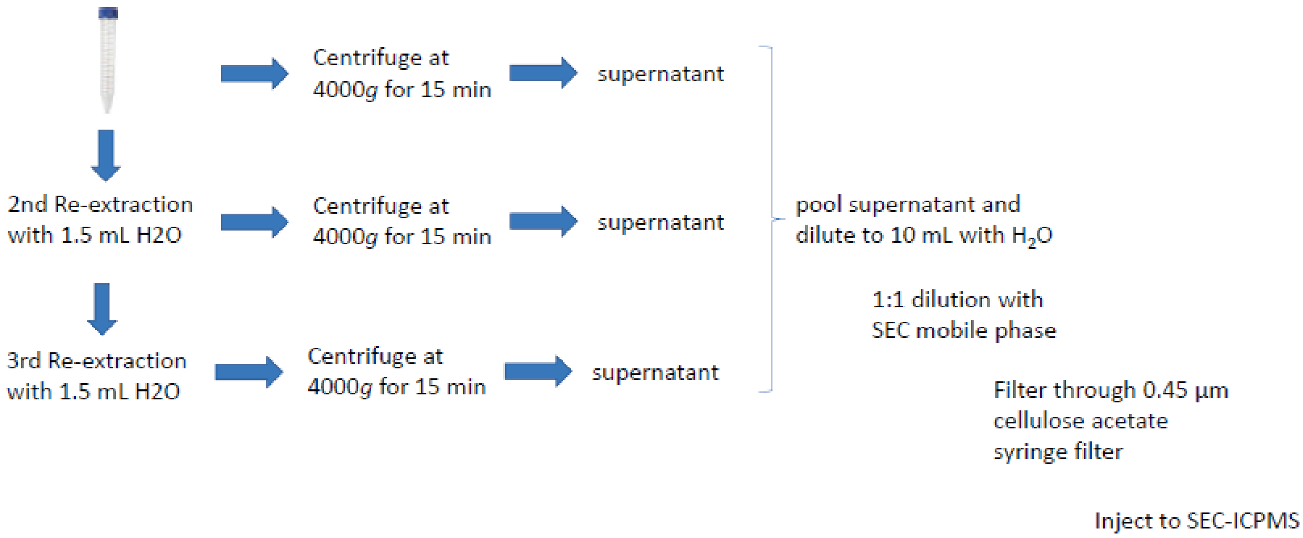

Figure 5. Ultrasonic treatment

Zinc: total measurements

Freeze dried yeast ⟶ Total Zn Digestion ⟶ Measured of total zinc by ICP-OES

Microwave accelerated digestion was perform with CEM MARS 5:

- ✓ 0.1g Lyophylised sample
- ✓ 5 ml 65% HNO_3
- ✓ Leave 3 days at room temperature in PTFE digestion vessel
- ✓ 3 ml 30 % H_2O_2
- ✓ Microwave digestion
  - 0-20 min ⟶ atm to 250 psi
  - 20-35 min⟶ hold at 250 psi
  - Cool to room temp
- ✓ Dilute to 25 ml with H_2O

ICP-OES method – was measured with Perkin Elmer Optima 8000

- Plasma gas: 15L/min
- Nebulizer gas:0.6 L/min
- Plasma power 1300 W
- Plasma view: radial
- Sample flow rate: 1.5 ml/min
- Wavelengh:Zn 213.858nm

The result of a previous study [16] was 14g/l yeast biomass with an enriched content of 1143.4 mg l^{-1} zinc.

The best results are shown for batch 2, concerning the total zinc and organic zinc of the first stage of the optimization obtained in this study are presented in table 2. It is observed that assimilated 1100 total zinc mg/kg in the yeast biomass were assimilated.

The chromatogram for the sample of yeast with zinc addition illustrating the signal of inorganic zinc can be seen in figure 6.

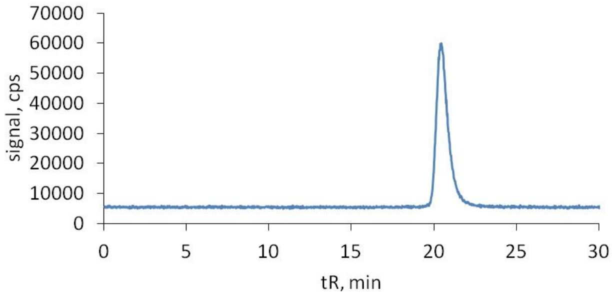

Figure 6. The chromatogram for the sample of yeast with zinc addition illustrating the signal of inorganic zinc

Table 2. Results for batch 2

total zinc mg/kg	inorganic zinc mg/kg	organic zinc mg/kg
1100	680	420

The yeast consortium biomass enriched with zinc was mixed with spent brewing yeast in the wet form, obtaining a new bioingredient enriched with zinc, which was taken into study as bioingredient for laying hens feed. In the study conduct by Anil Shet [12], two different enrichment methods were used to incorporate zinc into *Saccharomyces cerevisiae* biomass. The maximum amount of zinc in yeast cell was found to be 1699 µg/g and 1724 µg/g for a zinc concentration of 1.432 g/l in medium during growth and non-growth phase respectively. [12]

The results regarding the concentration of zinc from spent brewing yeast biomass (control) and mixed biomass samples obtained in our study are shown in table 3:

Table 3. Analyzes results for mixed batches

Analyses/Samples	Control -spent brewing yeast	(Batch Mix 1)
total Zn content, mg/kg	68	89
mg/kg yeast organic zinc	48-68	69-89
inorganic zinc mg/kg	<DL	<DL

The detection limit (DL) of inorganic zinc measurents is 20 mg/kg yeast sample."

The signal of inorganic zinc can be seen in Figure 7.

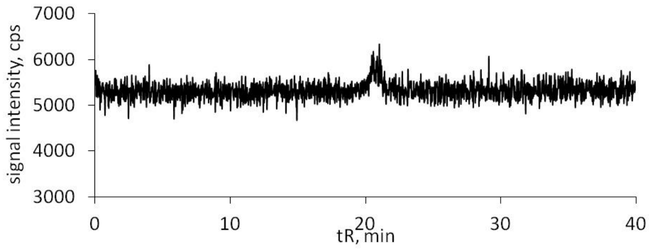

Figure 7. Chromatogram of mixed yeast samples (without zinc addition)

In table 3 and figure 7 the new product batch mix 2 can be observed, obtained by blending spent brewing yeast with zinc enriched with zinc. The concentration of total Zn content, mg/kg is 90, higher than that of spent brewing yeast, 22 mg/kg.

3. CONCLUSIONS

This product, based on the blend between spent brewing yeast resulted from the brewing industry with wet yeast biomass enriched in zinc organic is considered as an innovative feed supplement for laying hens.

Therefore, this is a challenge for animal nutrition specialists, to introduce and promote alternative feed resources that have high nutritive value.

Moreover, the valorisation of spent brewing yeast will be very important for the environment as it was considered to be waste.

As a first stage of optimization, a high concentration of total zinc at pilot level for the batch 2 and 3 was 1100-1760 mg/kg yeast, and 420 – 720 organic zinc mg/kg yeast.

The content from batch mixed 2 was: 90 mg/kg yeast total zinc and 70- 90 mg/kg yeast organic zinc.

For comparison, control samples (spent brewing yeast) the analyses shown a content of 68 mg/kg yeast Total Zn and 48-68 mg/kg yeast Organic Zn.

The Zn enriched yeast biomass was the subject for development of feed receipts for laying hens.

4. ACKNOWLEDGEMENTS (HEADING 1)

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