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CONSTRUCTION OF RECOMBINANT YEAST (*Saccharomyces cerevisiae)* PRODUCING β(1.3) GLUCANASE AS A FISH FEED ADDITIVES

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Abstract
The main goal of the feed industry is to manufacture the high quality food economically by increasing the biological value of the feed proteins. An addition to that, it is to provide higher quality animal products in sufficient amounts while keeping the input costs at optimum levels. Keeping these goals in our mind, in this study, the pRS416G recombinant vector carrying the β(1.3) glucanase enzyme, which has indirecting effects on the immune system significantly was transferred to Saccharomyces cerevisiae using the electroporation method. PCR reaction where Saccharomyces cerevisiae/pRS416G DNA is used as a template was examined on %0.8 agarose gel yielding approximately 1.9 kbç DNA band carrying the β(1.3) glucanase gene was visualized. After a zymogram analysis, the yellow zone expressed by β(1.3) glucanase gene was observed in the recombinant yeast extracts. This study will be pioneer work for the development of recombinant single cell proteins, thereby including immune stimulant of the β(1.3) glucanase as fish feed additives.

Key words: *Electroporation, Cloning, pRS416G, Saccharomyces cerevisiae, β(1.3) glucanase.*

INTRODUCTION

Increasing world population and standard of living necessitate the consumption of higher amounts and quality of food derived from animal products. Therefore, the proper feeding of the animals and improvement of animal genetic structure application of feed additives, such as antibiotics, enzymes, growth agents that mimic the effects of hormones, to improve the condition and productivity of livestock have an important role in fulfilling these requirements (Kaya et al., 1997).

It is well known that the use of antibiotics as a feed additive has positive effects on growth and on conversion ratio feed. However, antibiotic residue might occur due to excessive antibiotic use as a feed additive (Keser and Bilal, 2008). This residue may cause food poisoning in humans. For example, in poultry, pathogenic microorganisms like *Salmonella* and *Campylobacter* might develop multiple antibiotic resistances on animal bodies over the time. If animal products contaminated with these resistant pathogenic microorganisms are consumed by humans, food poison will be threated public health. As a result, the use of antibiotic growth factors was completely banned in the European Union after January 1st, 2006 (Keser and Bilal, 2008). Following this ban, studies to develop alternative feed additives for maintaining the digestive system ecology in balance and controlling enteric the bacterial diseases by reinforcing animal immune systems.

In recent years, enzymes as alternatives to antibiotics have increased. Xylanase, β-glucanase, pectinases, cellulases, proteases, lipase, phytase, galactosidase, β-mannanase and similar enzymes are used in the feed industry alone or in combinations with each other. The use of enzymes increases the digestion rates of the feed as well as yielding some immune stimulants on the feed (Karademir and Karademir, 2003).

In livestock farming, the main input that determines productivity and cost is the feed. In feed, feed stock with high protein content is heavily used. In fish feed, fish flour is an indispensable source of protein due to its high protein content, balanced aminoacid composition and its attractiveness for fish. However, due to reduced fish stock in recent years and its increasing use in human food, the manufacturing of fish flour has decreased, and feed manufacturers have started importing fish flour. As a result, the price of fish flour has increased feed costs and the use of plant based

sources came into consideration. In this context, various studies are being performed by fish feeders to reduce feed costs and to discover alternative protein sources and their usage conditions to replace fish flour Akyima et al., 1995; Webster et al., 1992; Wu et al., 1995).

It was determined that enzymes are used in various countries in recent years to increase digestion and as a result providing better live weight gain and feed benefit rate in feed manufactured using plant based stock (Karademir and Karademir, 2003).

Among these enzyme products, β(1.3) glucanes with indirect immunostimulant property for culture fisheries may be useful. It was reported that the fish became more resistant to various bacterial, viral and parasite related diseases, the mortality rates due to opportunistic pathogens in the larval stage has been decreased, increases were observed in the effectiveness of antimicrobial materials and growth rates and the negative effects of stress was reduced (Raa, 2000).

In fishes, the increase in the growth performance related to β(1.3) glucane is mostly related to maintenance of the health the feeding duration, environmental temperature and the studied species. It is reported that to achieve an increase in growth performance, feeding strategies must be developed for each fish species with regards to the dose and administration duration of β-glucane (Tonheim et al., 2008). As animals are exposed to stress factors every day, their immune systems weaken and this makes them sensitive against infections and other diseases. Animals fed with β(1.3) glucane containing feed have higher serum immunoglobulin levels and they become more resistant to infections and other diseases as a result. However, the addition of this enzyme to fish feed externally is very expensive, and it increases feed costs.

Insufficiency of feedstock that form the basis of the manufactured feed, and the decreasing amounts and increasing prices are the problem that must be overcome in culture fisheries (Arıman and Aras, 2002). In this regard, the addition of some enzymes or enzyme products to the feed imposes a very low cost. The prices of commercial enzymes are around 3-4 Euro/kg (9-12 TL/kg). Since in feed enzymes are used in very low ratios, around, 1-2 units per thousand, adding enzymes imposes a very small cost, around 8TL per 1 metric tonne of feed. Today, commercial enzymes for use in poultry feed are available on the market, but especially enzymes intended for use with fish feed have only recently appeared (Yiğit and Koca, 2011).

The goal of this study is to develop a recombinant *S. cerevisiae* strain that produces the bacterial β(1.3) glucanase enzyme as a source of single cell protein as well as feed addictive. This will allow the recombinant yeast to both provide single cell proteins (SCP) for growth source of the β(1.3) glucanase enzyme yielding β(1.3) glucan from yeast cell will in digestive tract of the fish to strength immune system.

MATERIALS AND METHODS

Yeast, Plasmid and Growth Environments

The pRS416G vector developed by Mazı et al. (2012) (Figure 1), the *Saccharomyces cerevisiae* yeast and the equipment used in this study are available in the Animal Biotechnology and Genetic Engineering Laboratory, Zootechnology Department, Faculty of Agriculture, Çukurova University. Chemicals were obtained from various companies. *Saccharomyces cerevisiae* was left to reproduce in YPD liquid medium overnight at 30˚C (Johston, 1994). The next day, the breeding yeast were planted into a solid medium containing antibiotics (ampicillin 50 µg/ml) against bacterial contamination with a sterile needle and this was left in YPD to incubate for another night at 30°C to colonise.

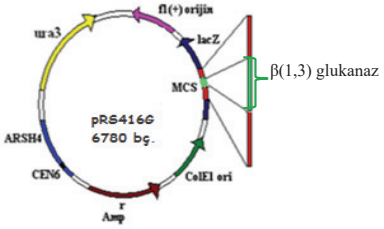

Figure 1. Map of pRS416G plasmid

Electroporation to Saccharomyces cerevisiae yeast

After breeding in the liquid medium, the yeast, after reaching the OD_{600}=1.3-1.5 value, was

made competent and electroporation was applied. Afterwards, the yeast was spread over minimal culture plates and left to incubate at 30ºC overnight to form colonies (Figure 2) (Özcan, 1992; Özcan, 2001).

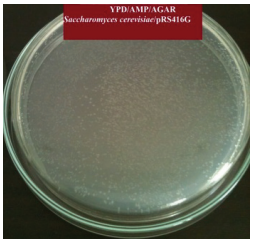

Figure 2. Yeast of *Saccharomyces cerevisiae*/pRS416G growth on YPD/Amp/Agar plates

DNA Isolation and PCR Reaction

pRS416G DNA was isolated from *Saccharomyces cerevisiae* (Harju et al., 2004). The primary string of the β(1.3) glucanase gene that is to be used for replication is based on 5'-AGAGCTCGTGGCACTGCACTCGTTCGAG TCT-3' (forward) and 5'-AGAGCTCGACGGGCGCGGTCA GAGCGTCCAG-3' (backward) gene strings (Shen et al., 1991). To detect the presence of the β(1,3) glucanase gene in the isolated DNA; a PCR reaction where pRS416G DNA is used as a template was prepared. The components of the prepared PCR reaction are as follows; 5 µL reaction buffer, 1 µL $dNTP_{mix}$ (200 µM for each), 1 µL each primary forward and backwards (20 pmol each primer), 0.5 µL Pfu DNA polymerase (2.5 U/µL), 1 µL DMSO 50% w/v (final concentration 1% w/v) and the total volume was adjusted to be 50 µL. The PCR program used was: at first 94ºC 2 minutes, afterwards 98ºC 30 seconds and 68ºC 5 minutes 30 cycles, afterwards 72ºC 5 minutes and kept at +4ºC. The result of the reaction was examined in 0.8% agarose gel with the help of marker DNA (Figure 3) (Özcan, 2001,).
SDS-PAGE experiments were performed (Laemmli, 1970). The bacteria culture was planted into 25 ml LB liquid medium with a sterile needle and left to incubate at 37ºC for 48 hours. At the end of this time *Saccharomyces cerevisiae* was drawn into 10 ml centrifuge tubes and precipitated by spinning it for 10 minutes at 4500 RPM. 5 ml from the upper phase were taken into clean centrifuge tubes; 1 volume 20% w/v TCA was added and homogenized with a pipette.

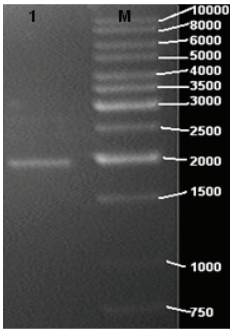

Figure 3. SDS-PAGE, Zymogram Analysis and Obtaining Extracellular Proteins through TCA Treatment

The mixture thus prepared (supernatant+TCA) was incubated at room temperature for 24 hours, and at the end of this time, the samples were put in a centrifuge for 10 minutes at 4500 RPM for the proteins to form pallets. The upper liquid part was poured away and the protein pellets were dried and then dissolved in 1 M Tris (pH 8). Zymogram analyses were modified based on Özcan (1992).

Display of Bands Responsible for 3.2. β(1.3) glucanase enzymes in SDS-Laminarin-PAGE

The gel was carefully removed from between two glass plates, transferred to the renaturation solution (80% v/v 50 mM Na-phospate solution (pH 7.2), 20% v/v isopropanol) and incubated in this solution for 1 hour. This way SDS was removed from the gel and proteins were renatured. The gel was removed from this solution and placed in 50 mM Na-phospate solution (pH 7.2) and left to incubate for 1 hour at room temperature. Afterwards the gel was transferred to 50 mM Na-phospate solution +5 mM β-mercaptoethanol + 1 mM EDTA solution and left to incubate overnight at +4ºC. The next day the gel was placed in sodium phospate solution and incubated for one hour at +4ºC; afterwards, the gel was removed from the sodium phospate solution, wrapped in cling wrap to prevent fluid loss and left to incubate at 30ºC for 4-5 hours. At the end of incubation the gel was dyed by the Congo-red dye for 1 hour. The gel was removed from the dye solution and washed in 1 M NaCl and 5 mM NaOH to

remove the excess dye, revealing the band responsible for the laminarinase enzyme that appears as a yellow zone (Özcan, 1992; Teather and Wood, 1982).

RESULTS AND DISCUSSIONS

Gene Expression results of Saccharomyces cerevisiae/pRS416G

Total proteins belonging to *Saccharomyces cerevisiae* were demonstrated in SDS-PAGE with Coomassie blue dye (Figure 4a). Protein samples were loaded into 12% SDS-Laminarin-PAGE (0.2% w/v laminarin) gel for the proteins to progress in the gel. After the protein progression in the gel is completed, the gel was treated according to the protocol given in 3.2 to display the band responsible for β(1,3) glucanase enzyme. The activity band belonging to the enzyme is shown by Congo-red dye in SDS-Laminarin-PAGE (Figure 4b) (Özcan, 1992).

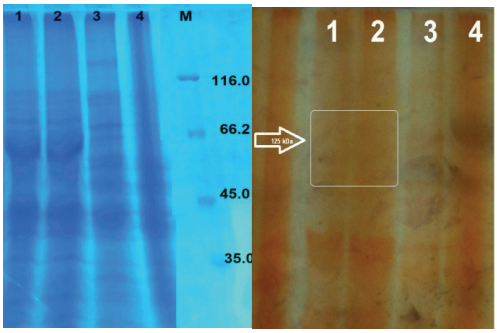

Figure 4. SDS-PAGE (a) and SDS-Laminarin-PAGE (b) analysis of total protein yeast of carrying the gene β (1.3) glucanase (M: Marker 1-2-3: Rekombinant yeast of carrying the gene β(1.3) glucanase4:Original yeast)

Today, the developments in biotechnology significantly benefit the primary goals of livestock farming, increasing productivity in amount and quality, thus increasing profitability. Among the biotechnological products that are presented to livestock farmers, enzymes, organic acids and probiotics are the most important alternatives to some problematic feed additives, with their natural sources, and their safety with regards to animal and human health. More studies and development on these products that can be used both for animal health and to improve productivity, and encouragement of their more widespread use, would make it possible for the livestock industry to reach larger profits (Karademir and Karademir, 2003). For this reason, in this study the pRS416G vector containing β(1.3) glucanase enzyme that has a significant place in the immune system was transferred to *Saccharomyces cerevisiae* using the electroporation method. Afterwards, through Zymogram analysis, it was determined that the molecular weight of the laminarinase (β(1.3) glucanase) enzyme was approximately 125 kDa (Figure 4b). Similar to the findings obtained, it was determined that the molecular weight of β(1.3) glucanase enzyme is 125 kDa (Fuchs et al., 2003). It was determined that the Endo-β(1.3-1.4) glucanase gene was the DNA part that carries the 1.4 kb *PvuI-ClaI* cutting enzyme recognition string during the cloning analysis of the Gram+ bacteria *B. subtilis*. To produce the plasmid that is formed as pEHB9 this gene was first cloned at the yeast's LEU2 area with the pJDB207 vector. Afterwards pEHB9 was transferred to *S. cerevisiae* and it was shown that the yeast synthesized endo β(1.3-1.4) glucanase. However, it was shown that the β(1.3-1.4) glucanase activity in pEHB9 was very low and only detectable (Hinchliffe and Box, 1984). Similarly the EXG1 gene that codes *S. cerevisiae* exo β(1.3) glucanase was cloned and expressed in yeast (Van et al., 1997). By appending the 2.7 kb DNA fragment that codes the *B. subtilis* endo β(1.3-1.4) glucanase gene from the *E. coli* plasmid pFG1 to the *E. coli*/yeast shuttle vector the hybrid plasmid YCSH was created. By transferring the hybrid plasmid to *S. cerevisiae* the *bgl S* gene was expressed. The expression level change of the *bgl S* gene in *S. cerevisiae* was 2.3 times and this is related to the 2.7 kb DNA fragment orientation. The enzyme substrate specificity and the pH optimum was determined to be similar to *B. subtilis* endo β(1.3-1.4) glucanase enzyme and it was noted that the *bgl S* gene expression level in *S. cerevisiae* was lower compared to *E. coli* (Chen et al., 2005). β(1.3) glucanes have an important niche as a feed additive. There are various studies that demonstrate that this enzyme is effective on the immune system of fish. Researchers have observed that there were changes in the fish performance when β(1.3) glucanase was used as a feed additive for large yellow croaker

(*Pseudosciaena crocea*) (Ai et al., 2007), *Pagrusauratus* species of bass (Cook et al., 2003), Nile bream (Whittington et al., 2005), *Oncorhynchus mykiss* (Sealey et al., 2008), carp (*C. carpio*) (Bogut et al., 1995). In another study, for 3 groups of broilers, in order, additive-free ration (control), antibiotic added ration and yeast β(1.3) glucane added ration were given. During the trial period, no significant difference was observed between the groups with regards to feed consumption and feed benefit. As a result, these researchers have stated that the lack of difference between the antibiotic group and the β(1.3) glucane group with regards to performance parameters can be considered an indication that β(1.3) glucane can be used as an alternative to antibiotics (Rathgeber et al., 2007).

CONCLUSIONS

This study aims to form β(1.3) glucanes that are very expensive to add to the feed from outside sources and that reinforce the non-specific immune system directly in the fish digestive system through *Saccharomyces cerevisiae*/pRS416G (Yeast cell wall + β(1,3) glucanase enzyme=β(1.3) glucane) for the purposes of reinforcing the fish immune system, improving resistance to various bacterial, viral and parasite-related diseases, preventing larval deaths due to opportunistic proteins and to reduce the negative effects of stress without using antibiotics. Also with the β(1,3) glucanase producing *Saccharomyces cerevisiae* strain, a new recombinant probiotic and/or single cell protein was developed.

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