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In-vitro digestibility studies with recombinant *Saccharomyces cerevisiae* expressing fibre degrading enzyme genes

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The ability of cattle and other small ruminants in digesting low quality fibrous feed is very important to improve their productivity. This fact is more significant in countries like India which has a very large cattle population and plenty of fibrous feed stuff. Cellulose is a major biopolymer which is present in plant matrix which needs to break down for efficient digestion. Cellulose has been reported to be degraded by three types of enzymes namely, exoglucanases, endoglucanases or cellobiohydrolases and β-glucosidases (Lynd *et al.* 2002). Continuous attempts are being made to isolate and characterize genes encoding novel enzymes from the rumen (Patel *et al.* 2016). Cellulases are a family of enzymes with the ability to improve the digestibility of fibrous animal feed. Majority of the current applications of cellulolytic enzymes are performed using small quantities of crude enzyme mixtures rather than purified enzymes wherein the hydrolysis efficiency of cellulose is very low and production cost of the enzyme is high. Isolation of microbial cellulases with high activity and generating genetically modified organisms are being attempted as an alternative strategy. Although, a large number of microbes are capable of degrading cellulose, only a few of these produce significant quantities of cell free enzymes capable of completely hydrolysing cellulose *in vitro* (Immanuel *et al.* 2006). *Phanerochaete chrysosporium* is the white rot fungus and known for its ability to produce cellulolytic and ligninolytic enzymes (Broda *et al.* 1995).

Endoglucanases (Avicelase) attacks regions of low crystallinity in cellulose fibre (Lynd *et al.* 2002). Exoglucanases catalyze the hydrolysis of beta- D-glucosidic linkages in cellulose with the release of cellobiose. Rumen simulation technique (RUSITEC) has been found to be an ideal system for *in-vitro* digestibility studies (Lengowski *et al.* 2016). The present study describes the *in vitro* digestibility of recombinant exo and endo glucanases by RUSITEC.

Phanerochaete chrysosporium culture obtained from IMTECH, Chandigarh, India was inoculated into potato dextrose agar plates followed by potato dextrose broth and used for RNA isolation. After 5 days of growth in potato dextrose broth, RNA was isolated from the mycelia of *P. chrysosporium* using Trizol reagent (Invitrogen, USA). cDNA was synthesized from the total RNA using Superscript first strand synthesis kit (Invitrogen, USA) and oligo dT primers. Endoglucanase gene was amplified with gene specific primers (FP: 5' CACCATGGCGAAGCTGTCGA 3' and RP: 5' CGAAGGGGCAGTCCCCTTGT 3') under the following cyclic conditions; 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1.5 min and a final extension of 72°C for 7 min. Amplification of exoglucanase gene was done with Exo FP 5'CACCATGGTCCGCGCCCGCCGCACT 3' and Exo RP 5' GTAGCACTGCGAGTAGTAAGG 3'. PCR products were checked by agarose gel electrophoresis and purified using gel extraction kit (Qiagen, Germany).

Gel purified exo and endo glucanase gene products were cloned into pENTR/D-TOPO vector (Invitrogen, USA). The ligated mixture was tansformed into *E.coli* DH5α cells and plated onto LB agar containing Kanamycin (25 µg/ml). Plasmid DNA was isolated from the recombinant clones to check for the presence of the genes and confirmed by restriction enzyme (RE) digestion. Positive clones were subcloned into pYES-DEST 52 by site specific recombination and transformed into *E.coli* Novoblue. Transformed cells were plated onto LB agar containing ampicillin (100 µg/ml). Recombinant pYES-DEST plasmids with exo and endoglucanase genes were confirmed by RE digestion and transformed into *Saccharomyces cerevisiae*. Recombinant cells were induced with 2% galactose and checked for expression by SDS-PAGE. Plate assays using positive clones were performed as per the method of Teather and Wood (1982) and transformants which produced clearing zones, indicative of hydrolysis of

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cellulose were selected.

In vitro trials were conducted to study the effect of recombinant clones on degradation of lignocellulose and a thirteen day trial was carried out using RUSITEC unit consisting of four fermenters, following the general incubation procedure (Czerkawski and Breckenridge 1977). Fermenters were incubated with solid and liquid inocula from cow and inocula with pellet of recombinant yeast containing endoglucanase and exoglucanase gene of varying concentrations (1 g, 2 g, 3 g and control). Each fermenter received 15 g dry matter/48 h (paddy straw and maize concentrate) of the corresponding diet fed into nylon bags of 100 µm pore size. A continuous infusion of artificial saliva, pH-8.4 (Mc Dougall 1948) @ 600 ml/day was maintained throughout the experiment. All procedures were conducted under CO_2, with the solutions maintained at 39ºC. Samples were collected every 24 h for the etimation of microbial protein, volatile fatty acids (VFA), bacterial count, ADF analysis and NDF analysis. Nylon bags from each fermenter was removed every 48 h and replaced by new feed bag to determine the disappearance of dry matter (*in vitro* digestibility).

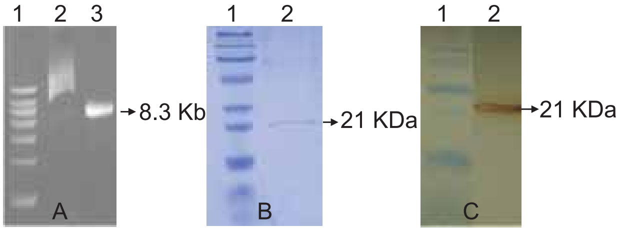

Fig. 1. Endoglucanase gene cloned in Yeast Expression vector pYESDEST-52 and protein expression analysed using SDS-PAGE electrophoresis followed by confirmation using western blot. A. 1% Agarose gel showing pYES-DEST52/Endo Clone digestion with *Not* I enzyme at lane 3. B. 12% SDS-PAGE analysis of recombinant protein in pYES-DEST induced with 2% galactose showing purified protein of 21 KDa in 12 h cultures (arrow). Lane1, Ladder; Lane 2, Endoglucanase enzyme protein. C. Western blot analysis showing 21 KDa protein. Lane 1, Protein Ladder; Lane 2: 21 KDa Endoglucanase enzyme protein.

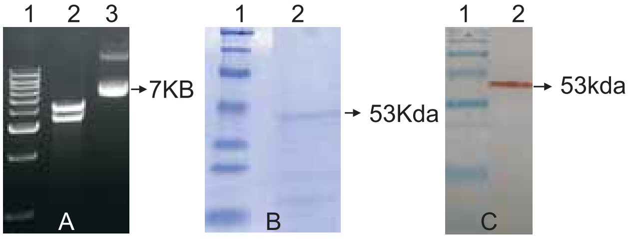

Fig. 2. Cloning and expression of exoglucanase gene in Yeast Expression vector pYESDEST-52. A. Agarose gel (0.8%) electrophoresis of Restriction enzyme digested recombinant pYES-DEST plasmid confirming the presence of exoglucanase gene. Lane 1, 1Kb ladder; Lane 2: cut with Enzyme *Nde* I; Lane 3: uncut plasmid. B. 12% SDS-PAGE analysis of recombinant protein in pYES-DEST induced with 2% galactose showing purified protein of 53 Kda in 12 h culture (arrow). Lane 1, Eluted sample 1; Lane 2, Eluted sample 2; Lane 3, Ladder. C. Western blot analysis showing 53 Kda protein. Lane 1, Induced showing 53 KDa; Lane 2, Marker.

P. chrysosporium is known for its ability to degrade lignin and cellulose (Kirk and Cullen 1998). In the present study, we attempted to clone and express recombinant fibrolytic enzymes and studied their *in vitro* digesting ability. RNA was isolated from the fungus, cDNA was prepared and PCR amplification resulted in 680 bp endoglucanase gene. Fairly good cloning efficiency was achieved and more than 50 Kanamycin positive endoglucanase ligated pENTR/D-TOPO colonies were obtained on LB agar plates. EcoRV enzyme digestion resulted in a linearized plasmid of around 3.2 kb with the cloned endoglucanase gene. Sub cloning into pYES-DEST resulted in 6.3 kb linearized plasmid following digestion with NdeI confirming the presence of endoglucanase gene in the destination vector. Recombinant pYESDEST-52 clone carrying endoglucanase gene was transformed into *Saccharomyces cerevisiae*, the yeast expression system. Expression of the 21 KDa protein was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by Western blotting (Fig. 1). Further confirmation by cellulose plate assay resulted in clearing zone with the recombinant yeast. Similar approach, used for cloning and expression of exoglucanase gene resulted in the expression of 53 KDa recombinant protein (Fig. 2) with cellulolytic activity.

In-vitro RUSITEC trials indicated significant increase in total bacterial counts with 0.5 and 1.0 g of r*S.cerevisiae* containing exoglucanase gene at 24 and 48 h as compared to the control (Fig. 3). Similarly, increase in total bacterial counts were observed with the recombinant endoglucanase. However, increase in total microbial protein was noticed only with recombinant exoglucanase at 24 h. Variations in the microbial population of rumen will have an impact on the fermentation pattern and could contribute to the

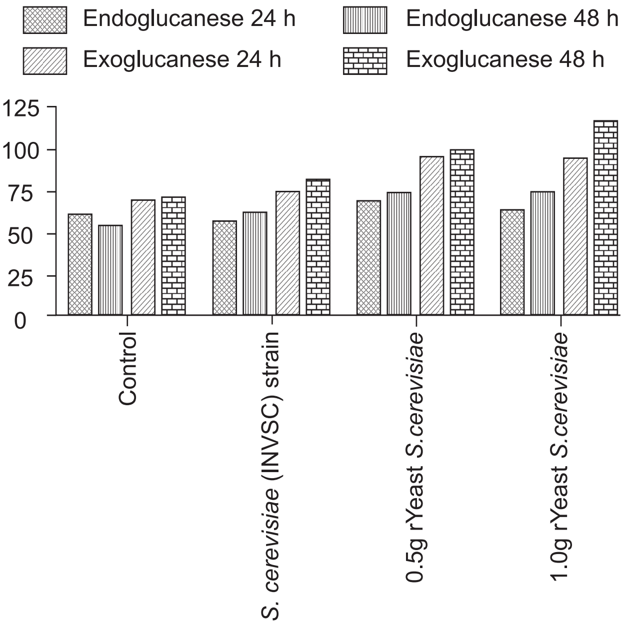

Fig. 3. *In-vitro* studies with recombinant exoglucanase and endoglucanase in RUSITEC estimating the total bacterial count

differences in the digestibility of ruminants. Higher bacterial count and low protozoan count is likely to result in increased fermentation thereby improving the digestibility (Wanapet *et al.* 2000). In our study, recombinant endoglucanase and exoglucanase increased the total bacterial count significantly at 24 and 48 h. Though both the recombinant clones increased the total microbial count, recombinant exoglucanase alone increased the total microbial protein. In accordance with the increased bacterial count, no significant increase in the protozoan count was observed in our study (Fig. 4). Generally, forages of low digestibility will result in the production of low levels of microbial protein in the rumen (Merchen and Bourquin 1994). Increasing the flow of microbial protein into the small intestine from the rumen will reduce the microbial protein requirement of the animal. In our study, recombinant exoglucanase increased the total microbial protein at 24 and 48 h (Fig. 5). This could probably due to increased production of the exoglucanase enzyme by the recombinant *S.cerevisiae* expressing the exoglucanase gene, increasing the availability of more enzyme in the environment of the rumen. However, no significant increase was observed with recombinant endoglucanase.

Though some of the studies with various exogenous enzyme products showed some degree of improvement in digestion, many did not affect the extent of digestion

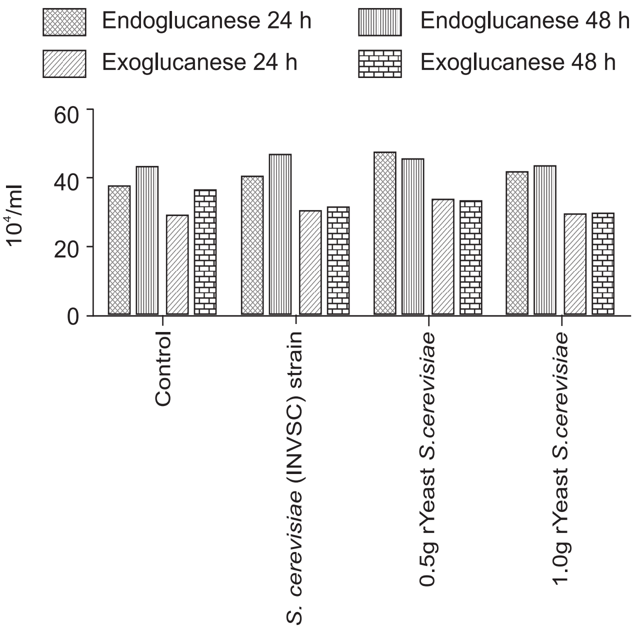

Fig. 4. Total protozoa count as estimated in RUSITEC with recombinant exoglucanase and endoglucanase

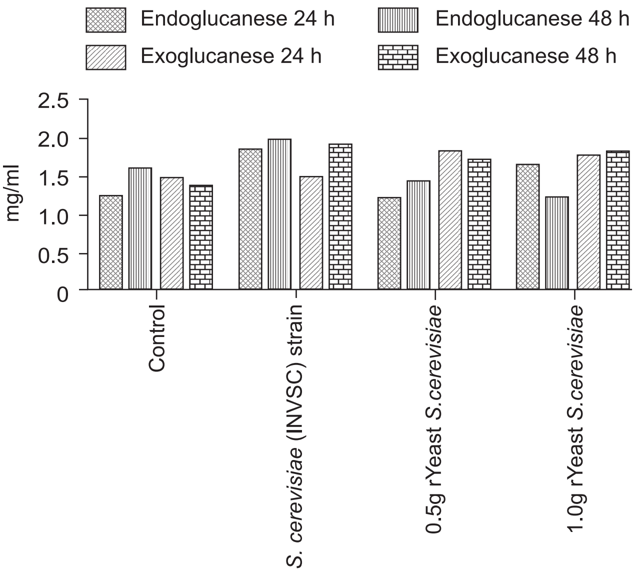

Fig. 5. Estimation of total microbial protein by RUSITEC method with recombinant exoglucanase and endoglucanase clones

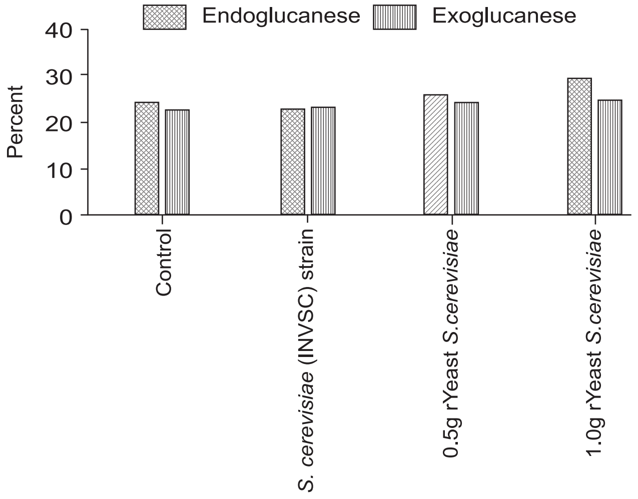

Fig. 6. *In-vitro* studies with recombinant exoglucanase and endoglucanase in RUSITEC assessing the *in vitro* digestibility

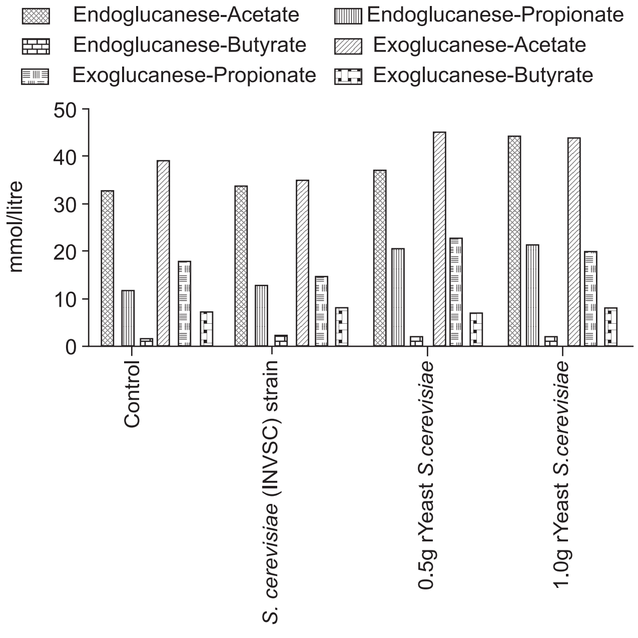

Fig. 7. Estimation of volatile fatty acids by *in-vitro* studies with recombinant exoglucanase and endoglucanase in RUSITEC

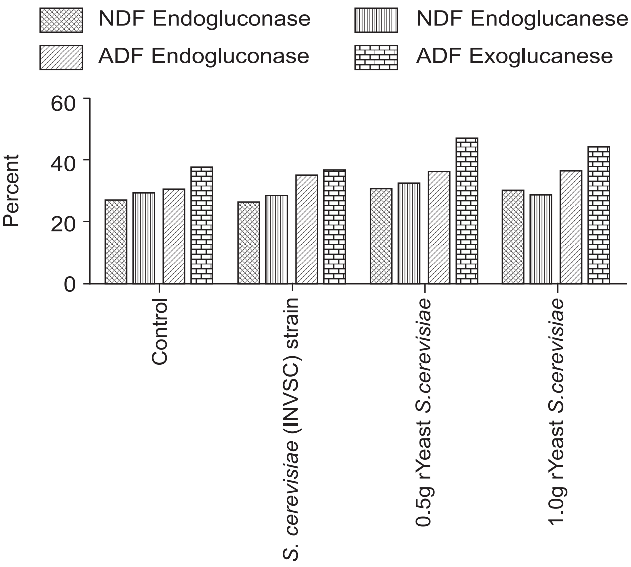

Fig. 8. *In-vitro* studies with the recombinant exoglucanase and endoglucanase in RUSITEC for NDF and ADF estimation

(Yang *et al.* 1999). In general, overall increase in the *in vitro* digestibility was observed with both endoglucanase and exoglucanase recombinants (Fig. 6). Digestibility was significantly higher with 1.0 g of recombinant endoglucanase than the exoglucanase.

When forage is consumed, volatile fatty acids (VFA) provide 50–85% of the metabolizable energy to the animal (Owens and Goetsh 1988). Acetic, butyric and propionic acids are considered as the products of anaerobic microbial fermentation. These are the source of nutrients and energy to the ruminants (Tagang *et al.* 2010). *S.cerevisiae* containing endoglucanase and exoglucanase genes resulted in a significant increase in propionic acid levels (Fig. 7). Similarly, both the recombinant genes produced significantly high levels of acetic acid than the controls. These results were in accordance with some earlier reports (Lewis *et al.* 1996) wherein use of exogenous fibrolytic enzymes had increased VFA levels. The NDF and ADF percentages increased with 0.5 g and 1.0 g of r*S.cerevisiae* containing exo and endoglucanase gene as compared to the controls (Fig. 8). Out of the two levels tested (0.5 g and 1.0 g), the NDF and ADF levels were comparatively less at the higher concentration (1.0 g). Probably if the concentration of recombinant exo- and endo-glucanases are increased, the digestibility of NDF and ADF may also increase, which needs further investigation. It is concluded that recombinant *S.cerevisiae* expressing exo and endoglucanase genes improved the digestibility and microbial protein to a limited extent under *in-vitro* conditions. However, the *in-vitro* studies have been carried out only for a short duration and a detailed study is needed to assess the survivability of the recombinant organisms in the rumen environment which will be really challenging.

SUMMARY

The present study described the *in vitro* digestibility of recombinant exo- and endo-glucanase genes of *Phanerochaete chrysosporium* cloned into pYESDEST-52 vetor and expressed in *Saccharomyces cerevisiae*. *In-vitro* RUSITEC trials indicated that recombinant exoglucanase increased the total microbial protein at 24 and 48 h. Overall increase in the *in vitro* digestibility was observed with both endoglucanase and exoglucanase recombinants. Both the recombinants produced significantly high levels of propionic and acetic acid.

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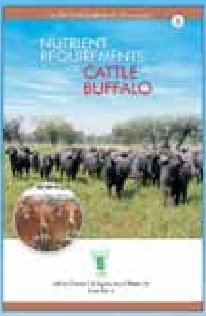
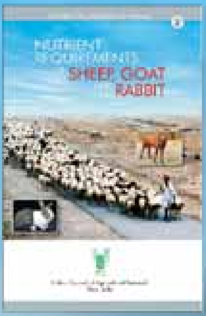
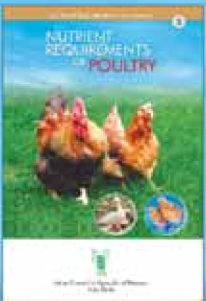
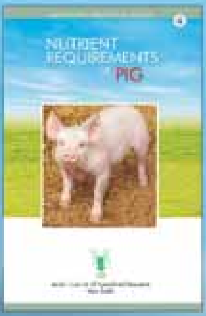
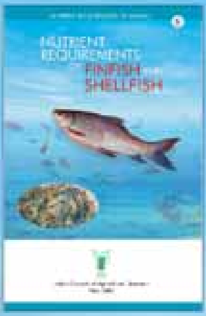
NUTRIENT REQUIREMENTS OF ANIMALS

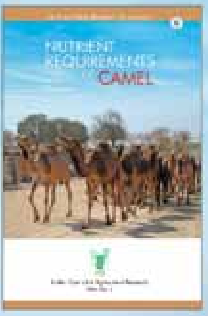


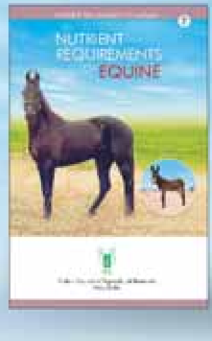


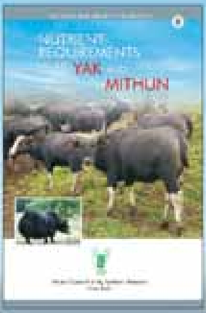


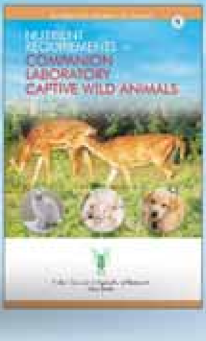


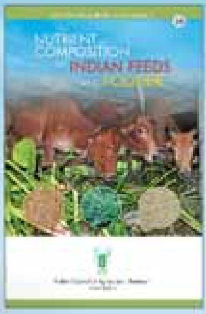












A nutritionally balanced 'livestock feed basket' improves the productivity of animals and simultaneously the economic condition of animal keepers. Feed requirement varies from species to species and from one geographic zone to another depending upon the animal potential and plant-soil-animal relationship. Several institutes of the Indian Council of Agricultural Research, have been working on these crucial aspects of animal nutrition since their inception. Earlier, ICAR published Nutrient Requirement of Livestock and Poultry in 1985 and 1998. Changing climate, vegetation cover and expectations of human population from animal resources have greatly affected the animal sector scenario. Realizing the fact that detailed information is required on nutrient composition of various feeds and fodders, the Council constituted a National Committee on Nutrient Requirements of Animals for compilation of information generated by these institutes.

In this present attempt the Committee has brought out 'Nutrient Requirements of Animals' – a series of ten publications. For the first time nutrient requirements of Camel, Yak and mithun, Companion, laboratory and captive wild animals besides Finfish and shellfish have been compiled. This series will be a must reference resource for livestock policy-framers, researchers, academicians, extension officials and grassroot farmers who steer positive changes in the societies' nutritional security and social integration.

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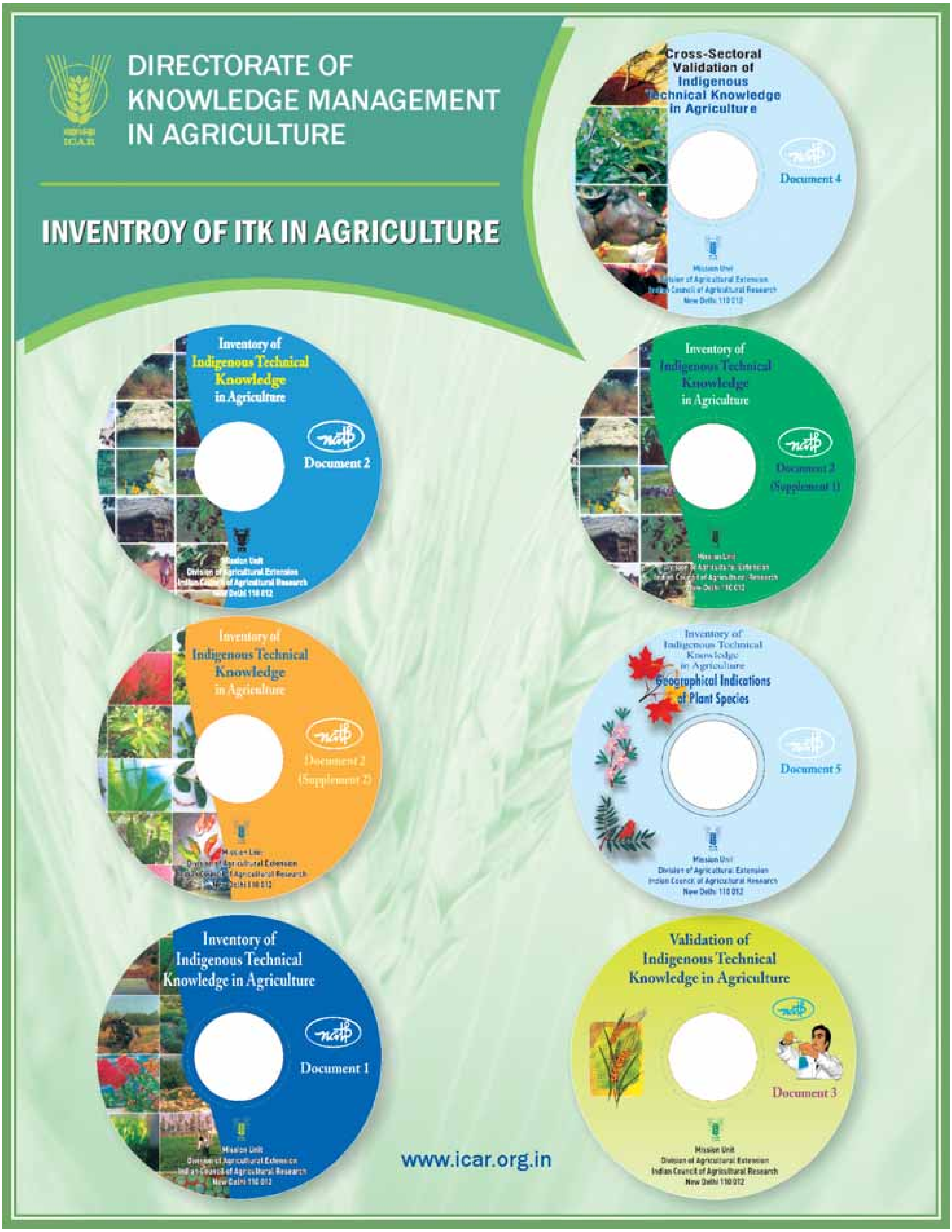

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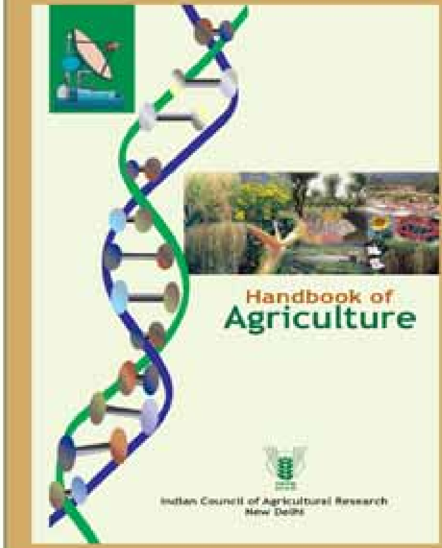
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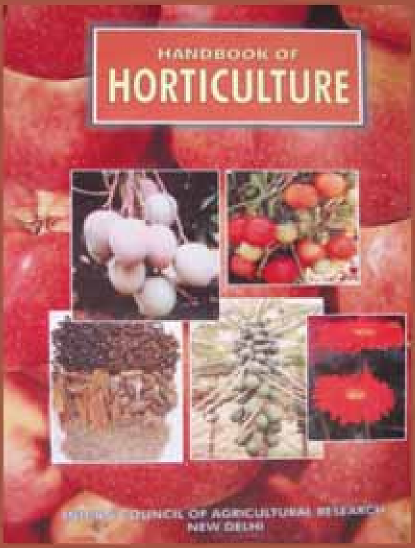
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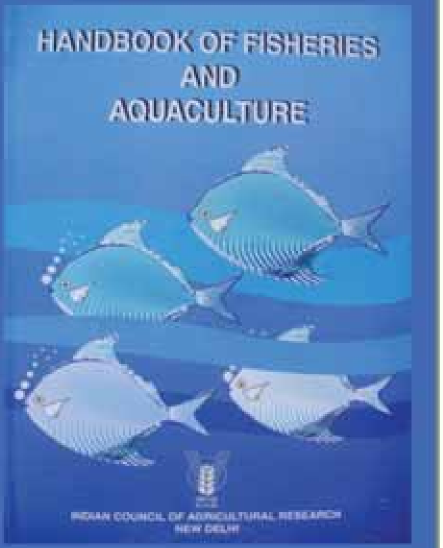
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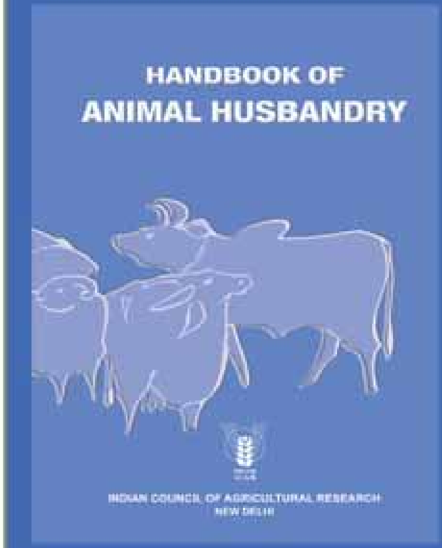
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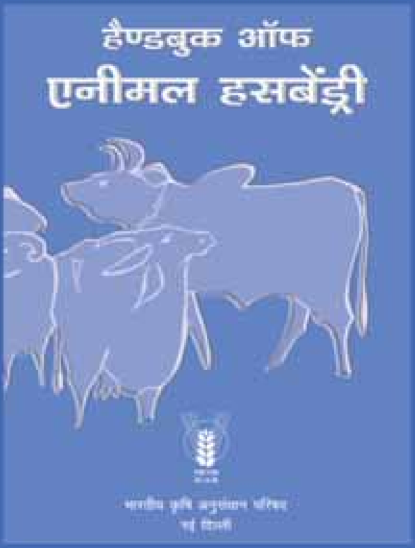
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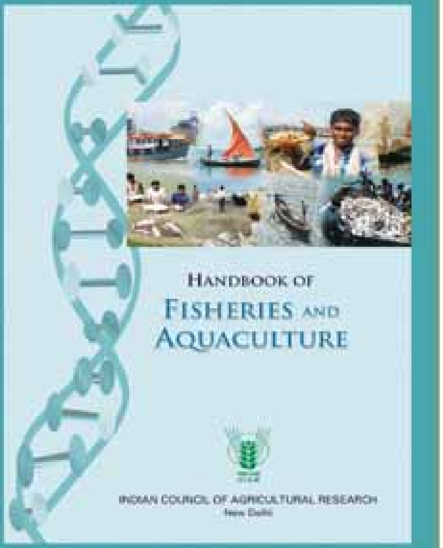
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