5. SUMMARY AND CONCLUSION

In the study entitled "**DEVELOPMENT OF ENZYME BASED HAIR DYEING PROCESS: APPLICATION OF AN ALKALINE BACTERIAL LACCASE FOR HYDROGEN PEROXIDE FREE HAIR COLORING"** the aim was to develop a chemical free, enzyme (laccase) based hair dyeing process.

An environmental isolate *Bacillus subtilis* DS was isolated, which produced a novel extracellular alkali-stable laccase (LAC-DS). Conditions were optimized to enhance the yield and purify the enzyme. LAC-DS was useful for industrial applications because of its properties such as wide range of pH, temperature, and stability in the presence of halides, metal ions and surfactants. Moreover its high catalytic affinity toward p-phenylenediamine (PPD) at alkaline pH made it a highly suitable candidate for its application in the process of hair dyeing. Conditions were standardized and enzyme based developer and dye to be used in enzymatic process was formulated. The complete enzyme based hair dyeing kit includes dye and developer formulation was designated as '**COLORZYME'**. 'COLORZYME' efficiently dyed the grey hair with good retaining ability without causing any damage to hair and skin. The process worked out to be economically viable in preliminary evaluations. The detailed summary of the study is as follow:

5.1 Selection and identification of the isolate

Isolation of laccase producing bacteria

- Soil/effluent samples were collected from environments where phenolic compounds were present in abundance and/or lignolytic material was decaying.
- Thirty nine laccase positive bacteria were isolated, out of which 2 were found to produce extracellular laccase.
- Enzyme from shortlisted isolates was able to oxidize laccase specific substrates such as ABTS and syringaldazine.

Selection of Isolate

- The selection of the isolate was carried out on the basis of high enzyme yield, effective oxidation of PPD in alkaline range at wide temperature.
- Isolate DS-1 was selected because of:
    - Effective oxidation of PPD.
    - Its activity in the temperature range of 45-60°C (optimum at 55°C and 50°C for SGZ and PPD as substrate respectively).
    - Its activity in the pH range of 6.0-9.0 (6.5 and 8.0 for SGZ and PPD as substrate respectively).

Identification of Isolate

Isolate DS-1 was identified and designated as *Bacillus subtilis* DS on the basis of following characteristics:

- Reddish brown, irregular, smooth, flat colonies on M162 agar plate.
- Gram positive, non-capsulated, sporulating rods, placed singly (sometimes in chains or bunches).
- Biochemical characteristics typical to that of genus *Bacillus*.
- 16S rRNA gene sequence (*MF359736)* analysis which revealed that organism phylogenetically belonged to the Genus *Bacillus* and was closely related to the type strain *Bacillus subtilis subsp. inaquosporium*.
    - Isolate was deposited in Microbial Type Culture Collection (MTCC), Chandigarh, India with MTCC number 12618.

5.2 Hyperproduction of laccase from *Bacillus subtilis* DS

Optimization by one variable at a time (OVAT) method

- A 26.46 fold increase in laccase production (1.1 to 29.46 $IUml^{-1}$) was achieved.

Optimization by statistical methods

- Placket-Burman design was employed to rank and choose significant variables.
  - Among 19 factors, ten factors viz. yeast extract >vanillic acid >tryptone >$MgSO_4.7H_2O$ > ferulic acid > $CuSO_4.5H_2O$, >$CoSO_4.7H_2O$ >$FeSO_4.7H_2O$ >methanol >xylose were found to have positive effect on laccase yield.

- Central composite design of Response Surface Methodology (RSM) was employed.
  - Interactive effect of yeast extract, vanillic acid, tryptone, and $MgSO_4.7H_2O$ was studied for the optimal production of laccase.
  - A 746 fold increase in laccase production (1.0 to 820.16 $IUml^{-1}$) was achieved.

These results have been published in Kumar D, Kumar A, Sondhi S, Sharma P and Gupta N (2018) An alkaline bacterial laccase for polymerization of natural precursor for hair dye synthesis. 3Biotech 8:182

  - cost analysis for the production Tentative per unit cost of enzyme under laboratory conditions was $6.06X10^{-4}$ Rs.
  - This cost was highly economical in comparison to other studies.

5.3 Purification and characterization of laccase from *B. subtilis* DS

Purification

- Enzyme was purified by first concentrating the proteins with 40-80% ammonium sulfate, then by passing it through DEAE-cellulose anion-exchange column and sephadex G-100 gel filtration column.
- After final purification step, enzyme was purified to 30.33 fold with a specific activity of 393.4 $IUmg^{-1}$ and 15.65% yield.
- Purified protein showed a single protein band of 34kDa on SDS-PAGE.
- Zymogram analysis of SDS-PAGE showed a reddish brown color of laccase activity at position corresponding to the purified protein band of 34kDa.
- The purified laccase was designated as LAC-DS.

Characterization of LAC-DS

Important characteristics of LAC-DS were:

- UV-visible spectrum (200-800nm) showed characteristic peak at 600 nm corresponding to the presence of Type 1 copper centre and a shoulder at 330nm corresponding to the presence of Type 3 copper centre.
- Optimal activity at 50°C and 45°C for SGZ and PPD respectively; more than 70% activity could be detected at temperature between 35°C and 70°C.
- 80 % activity/stability for 24h at all the temperature (45°C-70°C).
- pH optima for ABTS, SGZ, and PPD was 3.5, 6.5, and 8.5 respectively.
- Stability in the pH range 6.0-9.0 as it could retain more than >65% of the activity up to 5h. At pH 9.0, it could retain 40 % of activity even after 12h.
- Stability in presence of various halides and metal ions.
- Inhibition by most of the known inhibitors of laccase *viz.* cysteine, β-mercaptoethanol, EDTA and sodium azide.
- Stability in presence of various surfactants, but in the presence of CTAB activity was inhibited.
- High catalytic efficiency towards PPD and laccase specific substrates such as ABTS and SGZ.

5.4 Application of LAC-DS for hair dyeing

Standardizing for the optimal oxidation of PPD with LAC-DS

- **Optimization by one variable at a time (OVAT) method**
  - Optimum oxidation of PPD was achieved with enzyme dose of 10 $IUml^{-1}$, pH of 8.0, reaction time of 20 min and substrate concentration of 8mM at room temperature.

- **Optimization by statistical methods**
  - Optimized conditions after the statistical optimization were found to be enzyme dose of 12 $IUml^{-1}$, reaction time of 25 min, substrate concentration of 8.5mM and pH 8.0.

Dyeing of hair with enzymatically oxidized dye (PPD) in comparison to H_2O_2

- On dyeing of grey hair it was observed that performance of enzymatically (LAC-DS) oxidized dye was as good as that of dye oxidized with H_2O_2.

Structure analysis of hair dyed with enzymatically and H_2O_2 oxidized dye

- Fourier transform infrared (FTIR) analysis showed that spectrum of hair dyed with enzymatically oxidized dye was similar to that of control (Un-treated) hair. However changes in spectrum were observed in the hair dyed with H_2O_2 oxidized dye..
- Scanning electron microscopy (SEM) analysis showed that enzymatically oxidized dye caused no damage to the hair whereas damage to the hair was observed with H_2O_2 oxidized dye.

Retaining ability of dye oxidized by LAC-DS with respect to repeated washing

- Hair dyed with enzymatically oxidized dye were repeatedly washed with shampoo.
- There was less change in the color of hair upto 5^{th} washing gradual decrease the intensity of color was noticed with substituent washing.

Application of LAC-DS using commercial preparations

- **Hair coloring performance of commercial dyes oxidized with LAC-DS**
  - Grey hair were dyed with three commercial dyes (Commercial dye preparation-1,2 and 3) oxidized with LAC-DS instead of H_2O_2.
  - It was found that all the commercial dye preparations were oxidized by LAC-DS and they were able to give a smooth uniform black color to the hair.

- **Retaining ability of the commercial dyes oxidized with LAC-DS on hair with respect to repeated washing**
  - Hair dyed with enzymatically oxidized commercial dyes were repeatedly washed with shampoo. There was no lose of hair color even after 10 washing cycles.

FTIR analysis of hair dyed with commercial dyes oxidized with LAC-DS

- FTIR analysis showed that none of the enzymatically oxidized commercial dye caused any damage to the hair.

5.5 Development of enzyme based process for hair dyeing.

Formulation of dye to be used in laccase based hair dyeing process

- Ingredients which are generally used in commercial dye formulations were evaluated for their effect on stability/activity of enzyme for 6h at concentration of 0.01% and 0.1%.
  - Enzyme was found to be completely stable in the presence of most of the agents.
  - However enzyme stability/activity was highly effected in the presence of ammonium per sulfate (APS), o-phosphoric acid and sodium metabisulfite.
- Composition of final dye formulation was:
  - Phosphate buffer pH-8.0 (0.1M).
  - PPD at a concentration of 1.6%.
  - All other components at a concentration of 0.1%.
  - As before application, dye and developer were mixed in equal proportions final concentrations of PPD was 0.8% and all other components was 0.05%.

Formulation of laccase based developer for hair dyeing

- Ingredients such as ethanol, glycerol and cetryl alcohol, which are generally used in commercial developer formulations were evaluated for their effect on stability/activity of enzyme for 30 days at concentration of 0.01% and 0.1%.
  - Enzyme was found to be completely stable in the presence of all the agents.
- Composition of final dye formulation was:
  - Phosphate buffer pH-8.0 (0.1M).
  - Enzyme 12 $IUml^{-1}$.
  - All other components at a concentration of 0.1%.
  - As before application, dye and developer were mixed in equal proportions final concentrations of enzyme was 12 $IUml^{-1}$ and all other components was 0.05%.

Enzyme based hair dyeing kit

- The complete enzyme based hair dyeing kit including dye and LAC-DS based developer formulation was designated as **'COLORZYME'.**

'COLORZYME' enzyme based hair dyeing kit (Prototype label)

5.6 Dyeing of hair with 'COLORZYME' and its comparison with commercial H_2O_2 based process

Hair coloring performance

- Hair were dyed with 'COLORZYME' and commercial H_2O_2 based process (commercial dye and developer preparation-1); dyeing performance of hair with 'COLORZYME' and commercial H_2O_2 based process was comparable.

Retaining ability of hair color

- Hair dyed with 'COLORZYME' and commercial dye were repeatedly washed with shampoo.
- Retaining ability of color with enzymatic process was as good as that of commercial process
- There was little change in color of hair upto 15^{th} washings and gradual decrease in the intensity of color was observed with subsequent washing.

Structural analysis of hair

- Structural analysis by FTIR
  - FTIR spectrum of hair dyed with 'COLORZYME' was similar to that of control (Un-treated hair) the only difference was minor changes in the intensity of some of the peaks.
  - However gross changes were observed in the spectrum of hair treated with commercial H_2O_2 based process.

- Structural analysis by SEM
  - SEM analysis showed that 'COLORZYME' oxidized dye caused no damage to the hair whereas severe damage to the hair was observed with H_2O_2 oxidized dye.

- Structural analysis by SAXS
  - SAXS analysis of hair dyed with 'COLORZYME' was similar to that of control (Un-treated hair).
  - Whereas there were visible changes in intensity of some of the peaks in case of hair dyed with commercial process, indicating the damage of the hair protein/s by H_2O_2.

5.7 Toxicity evaluation and assessment of commercial evaluation of 'COLORZYME'

Cytotoxicity evaluation of 'COLORZYME' in comparison of commercial H_2O_2 based process

- Cytotoxicity evaluation on skin;human melanoma cell line
  - No significant effect in cell viability was observed with any component of 'COLORZYME'.
  - However 10% - 12% decrease in cell viability was observed with H_2O_2 based process.

- Cytotoxicity evaluation on macrophage (Raw 264.7) cell line
  - No significant effect in cell viability was observed with any component of 'COLORZYME'.
  - However 11% - 13% decrease in cell viability was observed with H_2O_2 based process.

Shelf life of enzyme based developer

- Shelf life of enzyme based formulation was evaluated at room temperature for one month in terms of stability of laccase in the developer.
- Enzyme was stable in the developer for extended period of time at both the temperature as about 97% of activity was retained even after 30 days of incubation.

Cost estimation of 'COLORZYME'

- To check commercial viability, approximate cost of one unit (50ml of developer + 50ml of dye) of enzyme based process was estimated under laboratory conditions.
- Final per unit cost of LAC-DS based process was approximately 58.7 INR.
- Although no actual comparison could be done but this cost was comparable with the cost of commercial dyes in the market.

5.8 Use of LAC-DS for synthesis of dye/s from natural precursors

Different natural precursors were used for the synthesis of dyes using LAC-DS at pH 8.5.

- Black color similar to that of PPD was developed using pyrogallol and catecol, golden yellow color was developed from ferulic acid and gallic acid, and reddish brown from syringaldehyde and syringic acid.

These results have been published in Kumar D, Kumar A, Sondhi S, Sharma P and Gupta N (2018) An alkaline bacterial laccase for polymerization of natural precursor for hair dye synthesis. 3Biotech 8:182

CONCLUSION

A novel extracellular alkali stable laccase (LAC-DS) was isolated from an environmental isolate *Bacillus subtilis* DS. Enzyme was hyper-produced, purified and characterized.

LAC-DS was used to develop **'COLOZYME'**, a H_2O_2 free user friendly hair dyeing process. 'COLORZYME' has high commercial potential as it showed good hair dyeing performance and retaining ability of color on hair. Moreover enzymatic process was better than commercial H_2O_2 based process as didn't cause any damage to hair and skin. Cost of

'COLORZYME' was found to be economical viable and its extended shelf life make it suitable for commercialization. .

LAC-DS was also found to produce wide range hair colors by polymerization of natural precursors.

Commercialization of '**COLORZYME**' is being explored.