

## **Enzymatic Removal of Melanin in Enzyme Based Dehairing and Fibre Opening**

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### **Abstract**

Melanin is a natural pigment of skin and hair, which provides a protective function against sunlight. The non-removal of pigments from the skin gives the finished leather a patchy appearance. As leather industry is undergoing a paradigm shift towards bioprocessing, enzyme based dehairing and fibre opening are becoming ecologically important. However, the enzymatic dehairing and fiber opening of buff calfskins from certain origin results in non-removal of pigments. In this study, an attempt has been made to remove the pigments from buff calfskins using enzymes. The presence of melanin in buff calfskins was identified through UV-visible spectral analysis. Preliminary trials have been carried out with various concentrations of xylanase in enzymatic dehairing and fibre opening. Addition of xylanase provides complete removal of melanin during enzymatic dehairing as well as fiber opening. Semi-technical trials have been performed by employing xylanase during enzymatic dehairing and fiber opening individually. The removal of melanin was found to be 100%. The performance characteristics of the resulted leathers have been analyzed and found satisfactory.

## **Introduction**

The do-undo methods adopted in conventional leather processing generates huge amount of pollutants, in view of the fact that they subject the skin/hide to wide variations in pH. Pretanning and tanning processes alone contribute to more than 90% of the total pollution generated in a tannery. Leather industry is undergoing a paradigm shift towards bioprocessing. Present day innovations in biotechnology have proved that enzyme assisted dehairing using proteolytic enzymes with low amounts of lime and sodium sulfide is commercially feasible. Recently, a lime free enzymatic dehairing process along with reduced amount of sodium sulfide has been standardized for cowhides, which ensures complete dehairing within 18 hrs.<sup>1</sup> An enzyme only dehairing method for goatskins without the use of lime and sodium sulfide has also been established.<sup>2</sup> The interfibrillary proteins, which are mostly mucoids that contain carbohydrate as prosthetic groups, are removed during fibre opening. These non-collagenous proteins are known as proteoglycans. Hence, in principle, it should be possible to produce pelt by removing the protein-carbohydrate conjugates through the action of substrate specific enzymes. It has been shown that  $\alpha$ -amylase has specific activity on carbohydrate-containing proteins such as proteoglycans. Thanikailvelan et al have developed the enzyme-based fibre opening for cowhides using  $\alpha$ -amylase without using lime at pH 8.0.<sup>3</sup> Aravindhan et al have developed enzyme-based fiber opening process for sheepskins.<sup>4</sup> Usually, after the enzymatic dehairing and fibre opening the grain of the skin appears clean and white. However, in our early trials, similar enzymatic treatment on buff calfskins resulted in removal of hair with incomplete removal of epidermal layer leading to black or brown grain surface. This leads to patches in the surface of the final leather. This is primarily due to the presence of excess pigmentation as melanin in the buff calfskins. It is

reported that the solubilization of melanin occurs at a pH greater than 10.<sup>5</sup> The conventional liming method results in clean and white pelt due to the operational pH being greater than 10 during lime based processing. However, in the lime free enzyme-only methods, the removal of melanin is difficult during enzymatic dehairing and fibre opening, especially in buff calfskins. This is mainly due to presence of higher pigmentation in buff calfskins, which is difficult to be removed by solubilization of melanin at the operational pH of 8 during enzymatic dehairing and fibre opening process.

Melanin is a specific class of polycyclic biopolymer related to the humic acids and found throughout nature, importantly in humans and animals. Melanin is produced by the melanocyte due to tyrosinase action on amino acids. Melanin is a primary colour agent in hair, skin and eyes and it is believed to be a protective agent against the damaging effects of UV radiation.<sup>6</sup> There are three main types of melanins: 1) Eumelanins: brown black pigments derived from tyrosine following its conversion to dopa (dihydroxyphenylalanine); 2) Phaomelanins: reddish-brown pigments which are cystine derivatives of eumelanin and 3) Allomelanin: black pigments (similar to eumelanins) formed from catechols *via* polyhydroxynaphthalene. Eumelanins involve the formation of indole 5, 6-quinone by several steps. Melanin is then formed by polymerization. Melanins have the ability to readily undergo reduction and oxidation reactions due to its ability to accept or donate an electron very easily over a large pH range. Melanin is a negatively charged polymer mainly due to its numerous  $\text{-COO}^-$  groups. Therefore, it is likely that it can interact with positive charges on proteins. Thus, charge interaction between proteins and melanin polymer may be one mode of melanin–protein binding. This was tested using the zeta potential meter.<sup>7,8</sup>

Xylanase is an enzyme widely used for the bleaching of pulp and wood.<sup>9</sup> It hydrolyzes the xylan present in the material. Xylan is the most abundant non-cellulosic polysaccharide present in both hard wood and animal plants. Bio-bleaching of wood and pulps are well practiced in the industries employing xylanase.<sup>9</sup> Hence, the xylanase has been chosen to bleach the pigments present in the calfskins. It is classified under the category of hydrolases and it has acid catalytic behavior.

In the present study, the black material present in the buff calfskins were extracted and identified using UV-visible spectroscopy. Xylanase has been used during dehairing and fibre opening for the removal of pigment. The efficiency of removal of melanin by xylanase has been studied. The final quality of the leathers has been assessed through organoleptic and physical properties. Scanning electron microscopic analysis of leathers has been carried out and the results will be presented later.

## **Experimental Methods**

### **Raw Materials**

Wet salted buff calfskins from Indian origin (average weight of 3 kg per skin) were chosen as the raw material. Biodart (dehairing enzyme),  $\alpha$ -amylase (fibre opening enzyme) and xylanase (melanin removal) were procured from Southern Petrochemical Industries Corporation (SPIC) Limited, India. All chemicals used for leather processing were of commercial grade. The chemicals used for analytical techniques were of laboratory grade.

### **Analysis of Melanin**

The black material from the skin is removed by gentle scrapping after standardized enzymatic dehairing, which is described below. The black material was hydrolysed by using 1M NaOH in the presence of 3% H<sub>2</sub>O<sub>2</sub> in a boiling water bath for 30 min.<sup>10</sup> After cooling, the absorption spectra of hydrolysed matter were recorded in a Perkin Elmer Lambda 35 UV-visible spectrophotometer at room temperature in the wavelength range of 200-800 nm.

### **Standardization of Dehairing Process**

In order to standardize the application of dehairing method for buff calfskins, three methods of applications were chosen namely, dip and pile method, grain side application and drum method. Two soaked buff calfskins were used for each trail. The sodium sulfide and the dehairing enzyme concentration were fixed as 0.5 and 1% respectively. The depilatory composition for different application is given in Table 1. In dip and pile method, a solution of corresponding composition was made and the pieces were mixed with the solution for 10 min. Then the pieces were piled and left overnight. In grain side application, a thick paste

was prepared as per the composition and then applied on grain side, piled and left overnight. In the case of drum dehairing, the depilatory composition was added with soaked calfskins. The duration of treatment was 6 hours with 15 min running per hour. Subsequently the skins were left in the bath overnight. Next day, the calfskins were dehaired and rated on the basis of the average area without hair out of the total area.

### **Preliminary Trials on Pigment Removal**

#### **Effect of Pigment Removal During Enzymatic Dehairing**

Ten buff calfskins were soaked conventionally. Soaked weight of the each skins was noted. Two skins were used for each trail. The extent of removal of melanin during enzymatic dehairing by using xylanase was studied on soaked buff calfskins. The process for the xylanase treatment is given below.

<b>Process</b>	<b>Chemicals</b>	<b>% offer</b>	<b>Remarks</b>
Enzymatic dehairing (drum method)	Water	15	The duration of treatment was 6 hours with 15 min running per hour and left overnight in the bath.
	Biodart (SPIC)	1	
	Sodium sulfide	0.5	Next day, the extent of removal of pigment was assessed visually during manual dehairing.
	Xylanase	X	

X was varied as 0.1, 0.2, 0.3, 0.4 and 0.5%

Percentages based on soaked weight of calfskins.

#### **Effect of Pigment Removal During Enzymatic Fibre Opening**

Ten buff calfskins were soaked conventionally. Soaked calfskins were dehaired using standardized dehairing process. Two dehaired calfskins were used for each trail. The extent of removal of melanin during enzymatic fibre opening<sup>3</sup> by using xylanase was studied on dehaired buff calfskins. The process for the xylanase treatment is given below.

Process	Chemicals	% offer	Remarks
Enzymatic fibre opening	Water	100	
	$\alpha$ -amylase	1	
	Xylanase	X	Run for 3 hours. Extent of pigment removal was assessed visually.

X was varied as 0.1, 0.2, 0.25 and 0.3%

Percentages based on dehaired weight of calfskins.

### Optimized Process for Pigment Removal

Two optimized trials were selected based on the preliminary xylanase treatment trials for the removal of pigment during enzymatic dehairing and enzymatic fibre opening. Ten soaked buff calfskins were used for each optimized trial. The offer of xylanase was 0.5 and 0.3% for application during enzymatic dehairing and enzymatic fiber opening, respectively. In the case of xylanase application during enzymatic dehairing, the fibre opening was carried out using  $\alpha$ -amylase without employing xylanase.<sup>3</sup> **A control trial was performed without the use of xylanase** on ten soaked buff calfskins. The efficiency of removal of xylanase was assessed visually. Then the calfskins were chrome tanned using a conventional post-fibre opening process without deliming. The chrome tanned leathers were converted into upper leathers

using commercial post tanning process with the offer of 14% syntan, 10% fatliquor and 2% dye.

### **Physical Testing and Hand Evaluation of Leathers**

Samples for various physical tests from experimental and control crust leathers were obtained as per IUP method.<sup>11</sup> Specimens were conditioned at  $26.6\pm 2.2^{\circ}\text{C}$  and  $65\pm 2\%$  relative humidity over a period of 48 h. Physical properties such as tensile strength, % elongation at break and tear strength were examined as per the standard procedures.<sup>12,13</sup> Experimental and control crust leathers were assessed for **softness, fullness**, grain tightness, grain smoothness and general appearance by hand and visual examination. Two experienced tanners performed the assessment.

### **Results and Discussion**

The selection of raw materials has been based on the presence of higher amount of melanin in epidermal layer. It is known that buff calfskins contain higher amount of melanin as compared to other raw materials. Especially, buff calfskins from Northern part of India have the problems of non-removal of pigments during enzymatic dehairing and fiber opening. This study aims at complete removal of melanin during enzymatic dehairing and fiber opening.

### **Standardization of Enzymatic Dehairing for Buff Calfskins**

Trials have been performed in order to find the optimal application method for buff calfskins. The efficiency of dehairing for the various methods of application is shown in Figure 1. It is seen that the dip and pile as well as grain side application methods do not result in 100%

removal of hair. But, the drum method results in 100% removal of hair. This method of application is different from those observed for cowhides during sodium sulfide assisted enzymatic dehairing, where painting on grain side application<sup>1</sup> provided complete dehairing while dip and pile method<sup>14</sup> in the case of sodium metasilicate assisted enzymatic dehairing. This could be due to the structural and keratin-grain layer compaction difference between cow and buff calf species. Further studies were carried out based on drum dehairing method.

### **Identification of Melanin**

It is paramount important to ascertain that the non-removed material on the epidermis of buff calfskins after the enzymatic dehairing and fibre opening process is melanin. Hence, the non-removed material from the grain surface of enzymatically dehaired buff calfskins was extracted using standard procedure. The UV-Visible absorption spectrum for the extracted solution is presented in Figure 2. It is seen that the absorption maxima for the solution extracted from the grain surface of buff calfskins is 300 nm. It has been reported that the eumelanin shows absorption maxima at 300 nm.<sup>10</sup> Hence, it is evident that the absorption maxima at 300 nm for the extracted solution from buff calfskin is primarily due to the presence of eumelanin. Therefore, it can be concluded that the material present after the enzymatic dehairing and fibre opening is melanin.

### **Effect of Xylanase Treatment During Enzymatic Dehairing**

The extent of removal of melanin by xylanase treatment during enzymatic dehairing is shown in Figure 3. It is seen that the extent of removal of melanin increases with the increase in concentration of xylanase. The complete removal of melanin during enzymatic dehairing is

achieved at a xylanase concentration of 0.5%. The removal of melanin may be due to the action of xylanase on the adhering matter present between epidermal and melanin layer. The adhering matter may be such as proteoglycans or globular proteins. It has been postulated that the loosening of adhering substance containing glycans by xylanase results in the removal of melanin. Hence, the optimized concentration of xylanase for the removal of melanin is 0.5%.

### **Effect of Xylanase Treatment During Enzymatic Fibre Opening**

The offer of xylanase during enzymatic fibre opening was varied from 0.1 to 0.3%. The percentage removal of pigment by xylanase during enzymatic fibre opening is shown in Figure 4. It is seen that the increase in the concentration of xylanase results in significant increase in the percentage removal of melanin. At 0.3% offer of xylanase, melanin was completely removed. It is interesting to note that the 100% removal of melanin is achieved using low offer of xylanase in enzymatic fibre opening in comparison to enzymatic dehairing. This is due to the fact that substrate specificity for  $\alpha$ -amylase as well as xylanase is almost similar. Both the enzymes act on glycans, although their mechanism of action is different. Hence, the presence of  $\alpha$ -amylase assists xylanase in complete removal of melanin in spite of its low offer in comparison to during enzymatic dehairing.

### **Semi-Technical Trials for Melanin Removal Using Optimized Processes**

The use of xylanase during enzymatic dehairing and enzymatic fibre opening resulted in 100% melanin removal at an offer of 0.5 and 0.3%, respectively. These optimized processes were carried out at semi-technical level trials along with a control trial with ten buff calfskins

for each trial. The pelts from these processes were processed using conventional post-fibre opening process. The crust leathers were evaluated for strength and physical properties.

### **Strength Characteristics**

The resultant crust leathers from xylanase treatment during enzymatic dehairing and fibre opening were subjected to physical testing using standard procedures. The physical testing data is presented in Table 2. It is seen that all the strength properties of experimental leathers are comparable to the control leather values. Importantly, tensile strength of the experimental leathers is slightly better than the control leathers. All the strength values from control and experimental are meeting the standard norms.<sup>15</sup>

### **Organoleptic Properties**

The crust leathers derived from xylanase treatment during enzymatic dehairing and fibre opening were assessed for softness, fullness, uniformity in color, grain smoothness and general appearance. The data is presented in Figure 5. It is seen from that the leathers made from xylanase treatment have better rating in uniformity in color indicating that they are cleaner than control leathers. The grain smoothness is comparatively better than control leathers. Softness and fullness of xylanase treated leathers are comparable or even better than that of control leathers. The general appearance of the experimental leathers is much better than the control leathers.

### **Mechanistic Insight in the Removal of Melanin Through Xylanase**

Melanin is covalently linked to the skin protein. Degradation of melanin can be achieved by using oxidizing or reducing agents. On the other hand, breaking of bond between melanin

and the skin protein by employing suitable enzymes may induce melanin degradation.<sup>16</sup> In this study, it has been hypothesized that the removal of melanin may be due to the action of xylanase on the adhering matter present between the epidermal and the melanin layer. Loosening of adhering substance containing glycans by xylanase resulted in the removal of melanin. The catalytic activity of xylanase is based on a double-displacement mechanism.<sup>16,17</sup> It has been shown that the xylanases hydrolyze the  $\beta$ -(1,4) linked xylose backbone of xylans.<sup>17</sup> It is interesting to note that  $\alpha$ -amylase assists xylanase during its application in enzymatic fibre opening for the removal of melanin, which has almost similar mechanism. Two different mechanisms are suggested for the action of  $\alpha$ -amylase such as breaking the O-linkage between the protein and carbohydrate moiety or catalyzing the hydrolysis of the  $\alpha$ -(1,4) glycosidic linkages in glycans.<sup>18,19</sup>

## **Conclusion**

The non-removal of pigments from buff calfskins during enzymatic dehairing and fibre opening resulted leathers with unacceptable surface. In this study, this non-removed material was extracted from buff calfskin and identified as melanin using UV-Visible spectrophotometry. Optimization trials have been carried to find the optimal concentration of xylanase for its application during enzymatic dehairing and fibre opening. The complete removal of melanin has been achieved using xylanase during enzymatic dehairing and enzymatic fibre opening processes at 0.5 and 0.3%, respectively. Semi-technical level trials reveal that the crust leathers have similar or even better strength and bulk properties than the control leathers.

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**Table 1. Composition of depilatory mixture**

<b>Application</b>	<b>Water (%)</b>	<b>Na<sub>2</sub>S (%)</b>	<b>Enzyme (%)</b>
Dip and pile	15	0.5	1
Drum	15	0.5	1
Grain side	7	0.5	1

Percentages were based on soaked weight of buff calfskins.

**Table 2. Physical testing data of the crust leathers**

<b>Sample</b>	<b>Tear strength (Kg/cm)</b>	<b>Tensile strength (Kg/cm<sup>2</sup>)</b>	<b>% Elongation at break</b>
UNIDO norms <sup>15</sup>	30	200	60-70
Control without xylanase	31±2	246±12	67±5
0.3% xylanase during enzymatic dehairing	32±3	252±18	73±4
0.5% xylanase during enzymatic fibre opening	35±4	263±14	69±7

Average of mean of along and across backbone values for five leathers

## Figure Captions

**Figure 1.** Dehairing efficiency by various methods of application for buff calfskins

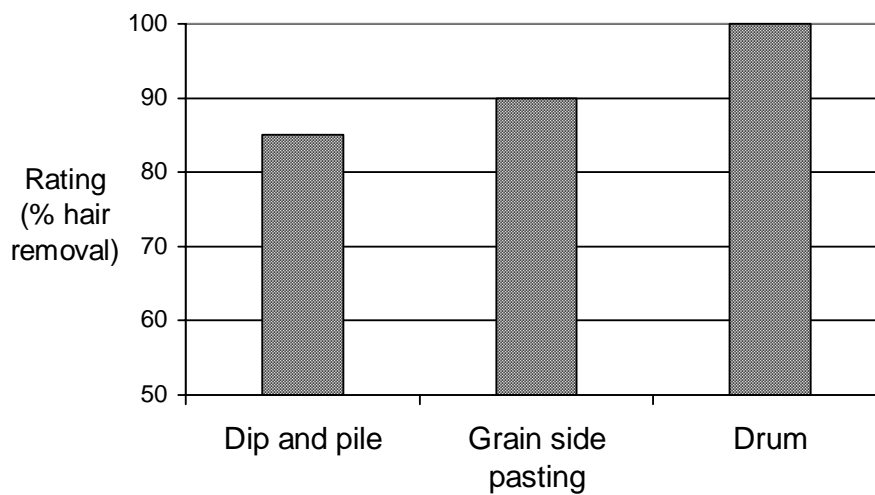
**Figure 2.** UV-Visible absorption spectrum of the extracted solution from the grain surface of dehaired buff calfskins using enzymes

**Figure 3.** Removal of melanin using xylanase during enzymatic dehairing

**Figure 4.** Removal of melanin using xylanase during enzymatic fibre opening

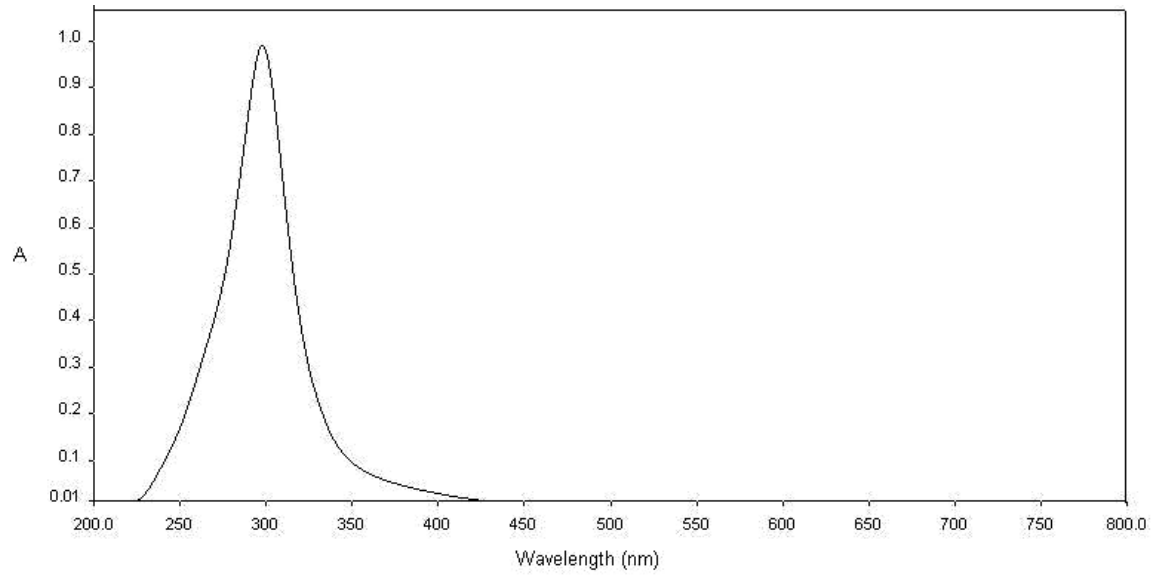
**Figure 5.** Organoleptic properties of crust leathers

**Figure 1.**



1% Enzyme and 0.5% sodium sulfide (based on soaked weight) was used in all the methods of application  
15% Water was used for dip and pile method  
15% Water was used for drum method  
7% Water was used for paste method

**Figure 2.**



**Figure 3.**

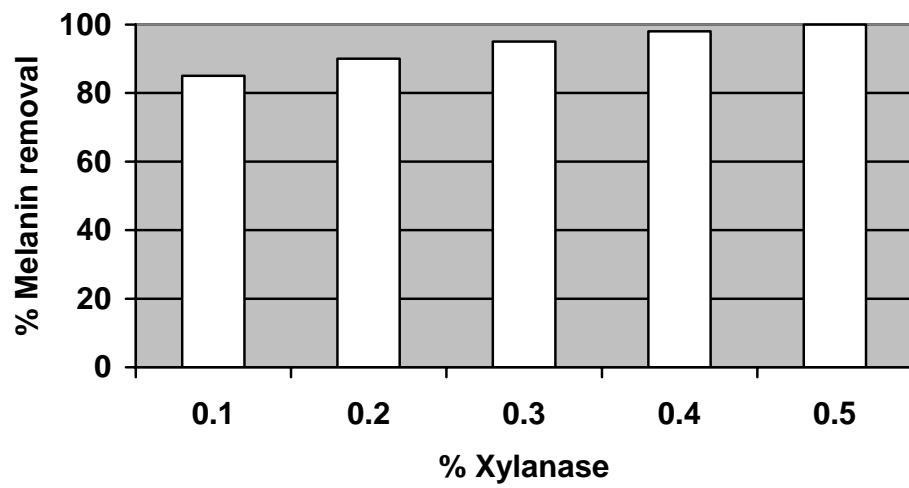


Figure 4.

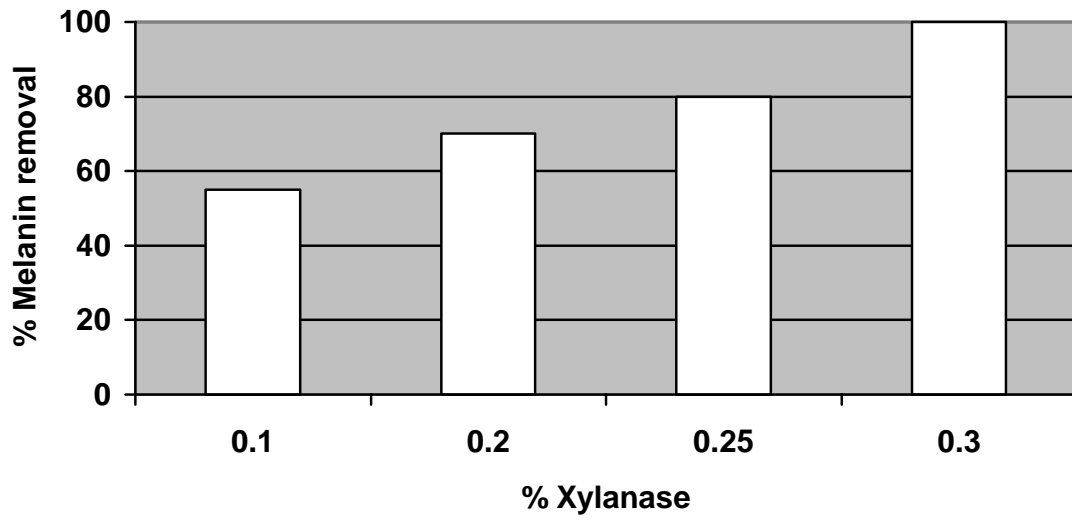


Figure 5.

