Comparative evaluation of various extraction methods on quantity and quality of Indigo dye from *Indigofera tinctoria* L.

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Abstract
An attempt was made to standardize the extraction of indigo from *Indigofera tinctoria* L. for maximizing the dye recovery and maintaining quality through various extraction methods. The experiment consisting factorial combinations of four levels of method of extraction *viz.,* conventional,microbial , chemical methods and hot water treatment and seven levels of duration of fermentation *viz.,* 0, 4, 8, 12, and 16, 20 and 24 h. The method of extraction showed significant influence on indigo content and dye yield. The highest dye recovery and indigo content were recorded with microbial fermentation method. The microbial method not only increased the yield and quality but also reduced the duration of extraction.

Keywords: Extraction methods, Indigo, Natural Dyes.

Introduction

Colour is a fundamental part of human perception and is an established aspect of civilization. Colour affects every moment of our lives. The addition of colourants to foods, textiles, cosmetics, pharmaceutical products is done to meet the demand of consumers and industry. Until the turn of 19th century all colours came from the natural world, as therewere no other means to derive them. With the discovery of synthetic dyes at the end of the 19th century, the cultivation and application of natural dyes disappeared.

Consumer's awareness about ecological liabilities of the textile dyeing industry and increasing strict effluent regulations has renewed an interest in natural dyes. In order to reduce thehigh pollution load which is characteristic of the modern textile dyeing processes, the partial replacement of synthetic dyes by natural ones in textile production may represent a strategy to reduce risks and pollutants. Hence, there is an increasing realization in the textile industry as well as among textile consumers to develop and demand eco-friendly methods of dyeing textiles. Natural dye offers an important alternative in this regard, as these are safer to use with minimum health hazards. They are easily disposable and biodegradable in nature.

Natural indigo derived from plant provides an ideal starting point for the reintroduction of naturally derived compounds into markets since there is already existing demand for the final product, mainly for dyeing cotton yarn for the production of denim (Gilbert and Cooke, 2001). Among the various dye plants cultivated, Indigo (*Indigofera tinctoria* L.) is an important and potential plant for commercial exploitation. The blue pigment indigo (indigotin) is one of the oldest natural dyes known to man. Indigo is not a native compound in plants, but is a product of a secondary metabolite named indoxyl. This metabolite is present in most of the indigo producing plants as indican (indoxyl-β-D-

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glucoside). Sarada and Reghunath (2006) have reported that the leaves have higher dye content (0.42 to 0.60 %) than in shoots (0.15 to 0.32 %).

The quality of dye depends on indigo content in the finished product after extraction. This has been reported to vary according to the methods used for extraction (Bechtold et al., 2002). The aim of dye plant cultivation should not only be high herbage yield but also a product with high content of valuable photochemical with very few foreign elements and free of toxic residues. (Wurl et al., 1997). The basic step in extraction of indigo involves fermentation of the plant followed by oxidation of the solution, boiling and filtration. Further, the quality of the dye is influenced by the duration of fermentation and oxidation. With this background, an experiment was formulatedto study the influence of various methods *viz.,* Conventional, Microbial, hot water and chemical methods to reduce the fermentation duration and to improve the dye recovery and indigo content in *Indigofera tinctoria* L.

Materials and Methods

Considering the importance of the crop, an experiment was conducted to standardize the extraction procedure for maximizing the dye recovery and quality of the dye through various methods. The experiment consisted of factorial combinations of four method of extraction *viz.,* conventional, microbial, hot water treatmentand chemical methods and seven levels of duration of fermentation *viz.,* 0, 4, 8, 12, and 16, 20 and 24 h. The experiment was carried out in plastic crates as vats of 3 cft size, one for fermentation and aeration tank and the other for settling.

Plants were harvested at 120 days after sowingleaving 15 cm above ground level. Ten kg of the plants were steeped in water in the fabricated extraction unit for 24 h. The steeping plants were then covered with a piece of wire mesh and immersed down with four stone blocks to hold the plant material under 10L of water. After 24h of soaking, extract along with the water was led into another tank of similar dimension and oxidized by agitating the green water by the aerator for 15 minutes. The indigo was allowed to settle for 24 h and the supernatant was siphoned off, leaving slurry of indigo and water. The sludge was transferred into a copper vessel and heated at 50^0C temperature for 45 minutes. Then it was filtered, pressed in a wooden press and dried (Anbalagan, 2005). Forthe microbial method, activated EM (effective microorganisms) procured from M/s. Mohan Microbes Ltd., Chengalpattu was mixed with the steeping water at a ratio of 1:300. The subsequent steps were done as per Anbalagan (2005). For hot water method (Stoker et al.,1998)., 8L of hot water (60^oC) mixed with cold water to make up 10Lto make up the temperature to 38^oC. Similar steps were followed as in the earlier methods suggested by Anbalagan (2005).

For chemical method, steeping the plants for 4h in a volume of 10L of water at 50-60^oC. The extracts were stored for 24h in the steeping tank. Concentrated ammonia solution (CA @ 0.67 ml l^{-1}) and calcium chlofied was added @ 1.5 g $litre^{-1}$. The indigo was allowed to settle for 24h before the supernatant was siphoned off, leaving slurry of indigo and water. The sediment was collected and filtered through a cotton cloth. After drying in a hot air oven at 70^0C and cooling to room temperature, the amount of crude indigo was weighed as suggested by Bechtold et al. (2002).

Observations on pH of the steeping vat water were recorded once in 4 hupto 24h to ascertain the rate of fermentation as per the procedure given by Bechtold et al. (2002) and Stoker et al.(1998). Colour of the steeping vat water was visually assessed by a panel consisting of twenty individuals using scaling technique. The scale ranged from grade 1 to 8, starting from colourless (G1), Light brown (G2), Brown (G3) , Light blue (G4), Bluish green (G5), Blue (G6), Dark blue (G7), Dark brown (G8) and the grades between 4, 5 and 6 being the best, 1-3 and 7-8 being the worst. From the quantity

of dye obtained from the 10 kg of feed stuff, dye recovery was estimated and expressed in percentage. Indigo determination was done by the method suggested by Chanayath et al. (2002). Eight milligram of the standard indigo was dissolved in 20 ml of conc. H_2SO_4 and diluted to 500 ml with distilled water. The solution was then diluted to different concentration *viz.,* 0.5, 1.0, 1.5, 2.0 and 2.5 ppm with the conc. H_2SO_4 solution (H_2SO_4: distilled water in the ratio of 1: 24) and the absorbance at 611 nm was measured to plot the standard curve. One gram of sample (indigo paste) was dissolved in 20 ml of H_2SO_4 and then diluted to 500 ml with a solution of H_2SO_4 and distilled water (1:24) and measured at 611 nm. From the standard values, the amount of indigo per gram of dye was calculated.

Results and Discussion

Unlike other natural dyes, indigo does not occur in the plant themselves, but is made during the extraction process from precursors. The precursors are compounds containing the indoxyl group. During extraction, the indoxyl is released and is spontaneously oxidized by atmospheric oxygen to indigo, the blue end product (Bechtold et al., 2002). The traditional method of producing indigo from *Indigofera* has been done by water extraction in three stages as described for the colonial period in Bengal (Perkin and Everest, 1918; Kumar, 2004 and Darrac and Van Schendel, 2006). In the first stage, freshly harvested plants are soaked in the steeping vat for about 24 h. The resulting solution formed due to anaerobic fermentation is then run off into a second tank, the beating vat. Air is introduced by manually kicking the water with legs for two hours. The oxidative conditions lead to the formation of a blue precipitate of indigo, which settles to the bottom of the tank. This is then run off as sludge into a container wherein the third stage, it is boiled to help purify the indigo, which is filtered, washed and finally dried in the form of cakes.

Though fermentation helps in the release of indoxyl, it has to be stopped at correct stage as the quality of dye from excessive fermentation would be poor when compared to good fermentation. The endpoint of fermentation is decided based on the change of colour of the steeping water or by taste of the liquid traditionally. Further, oxidation also plays a major role in the yield and quality of dye. Earlier studies to enhance the quality of the product to compete with the new marketed synthetic product recommended that good fermentation and immediate aeration would result in higher yield of indigo with good quality.

Influence of pH

During biomass fermentation, pH plays an important role in complete or partial fermentation of the samples. pH indicates the acidic or alkaline state of a solution, which represents the dynamic nature of reaction kinetics of biochemical reactions whether acidic, alkaline, amphoteric or neutral. This will affect indican release from plant biomass and thereby affect dye yield. In the experimental setups, at the end of each fermentation under different incubation period and temperature, pH of the broth was determined to understand the nature of reaction and role of microbes in the biochemical reactions, which regulates the leaf decomposition during the course of fermentation. It was reported that under abiotic stress changes in pH initiates apoplastic oxidative burst which leads to extracellular reactive oxygen species (ROS) production that can regulate reaction dynamics (Chung et al. 2008).

The data on change in pH and colour observed at various intervals of time (Table.1) reveals that the duration of fermentation has to be varied according to the method of extraction.The analysis of variance showed a significant influence of different methods of extraction and duration on pH of the vat solution. The interaction effect between extraction methods and duration of fermentation was also significant. Among the four methods of extraction, the lowest pH of 5.36 was observed in T_2 (microbial method), followed by T_3 (hot water method) with a pH of 5.73. The maximum pH of 6.48 was observed in T_1

Table1. Influence of various extraction methods and duration of fermentation on pH

Treatment No.	Method	Initial	Duration of fermentation							
			After Inoculation	4 h	8 h	12 h	16 h	20 h	24 h	Mean
T_1	Conventional method	7.5	7.50	7.25	6.70	6.40	5.99	5.90	5.73	6.48
T_2	Microbial method	7.5	6.10	5.83	5.78	5.31	4.89	4.85	4.80	5.36
T_3	Hot water method	7.5	6.20	6.40	6.48	5.50	5.30	5.16	5.10	5.73
T_4	Chemical method	7.5	6.66	6.56	6.37	6.26	5.88	5.75	5.60	6.17
Mean duration of fermentation		7.5	6.62	6.51	6.33	5.86	5.51	5.41	5.30	
S.Ed.CD (p=0.05)										
	Treatment		0.07				0.14			
	Duration		0.07				0.14			
	Treatment X Duration		0.14				0.28			

(conventional method). The duration of fermentation also showed significant differences among the treatments. A gradual decrease in pH was observed with increasing duration. Among the interaction treatments, the least pH of 4.80 was observed in T_2 (microbial method) at 24 h followed by 4.85 at 20 h of fermentation. This shows the conversion of indican to indoxyl in the vat solution during fermentation. Among the methods, T2 (microbial method) took only 12 h to reach the good fermentation stage judged by the bluish green colour of the vat solution, whereas T_1 (conventional method) took 20 h to reach the good fermentation stage. Further, correlation between the colour of the solution and pH was also noticed. It was also noticed that quality of the dye and dye recovery was highly influenced under the pH and colour. Under an optimal pH range of 4-5, superior quality indigo is obtained with highest dye recovery. Further, Laitonjam and Wangkheirakpam (2011) reported that after 3 days of incubation, pH of fermented solution of two indigo producing plants was 4.8.

The increase in the rate of fermentation over a short period (12 h) as observed in the T_2 (microbial method) may be due to microorganisms present in the EM culture. Plating studies revealed that the presence of lactic acid bacteria (*Lactobacillus plantarum* – 9.8 x 107 CFU/ml, *Pseudomonas fluorescens* (12.5 x 108 CFU/ml), yeast (*Saccharomyces cerevisae* – 7 x 108 CFU/ml) and actinomycetes (*Streptomyces griseous* – 8.2 x 108 CFU/ml) in the EM culture. Thus, the efficient microorganisms might have increased the hydrolysis of indican and facilitated its release from the leaves. The reduction in time might have resulted from more release of enzymes responsible for conversion of indican into indoxyl. Findings of Pathak and Datta (2009) corroborate the results of the present experiment. In addition, during indigo dye production microbes act upon plant cell that facilitate rupture of organelles leading to release of indican from vacuole and b-glucosidase enzyme from the chloroplast.This was supported by Higa (1992), Kyan and Higa (1997), Parr et al., (1997), Bolanas et al., (2005).

During fermentation and oxidation stage of indigo dye production, 12 h fermentation from the treatment T2 (microbial method) yields maximum dye corresponding to pH 6–6.8, thus the 12-h fermentation duration was nearly optimum for indigo dye production

Influence of Extract Colour

The colour of vat solution significantly varied due to various methods of extraction and duration of fermentation (Table 2).The change in colour of the vat solution was visually graded. The grade scale ranged from 1 to 8 (4, 5 and 6 being the best, 1-3 and 7-8 being the worst). Among the four methods of extraction, the highest colour score (5.1) was observed in T_2 (microbial method) followed by T_3 (hot water method) (4.8) and (3.9) in T_1 (conventional method), whereas the T_4 (chemical method) recorded the lowest colour grade of 3.5. Duration of fermentation also significantly influenced the colour development. The mean

Table 2. Influence of various extraction methods and durationof fermentation on extract colour of steeping vat

Treatment No.	Method	Initial	Duration of fermentation						
			4 h	8 h	12 h	16 h	20 h	24 h	Mean
T_1	Conventional method	1.0	2.1	3.4	4.2	4.2	5.6	6.2	3.9
T_2	Microbial method	1.0	4.4	5.3	5.6	6.3	6.3	7.1	5.1
T_3	Hot water method	1.0	3.2	3.6	4.5	5.7	7.2	8.4	4.8
T_4	Chemical method	1.0	2.8	3.3	3.8	4.1	5.4	7.2	3.5
Mean duration of fermentation		1.0	3.1	3.9	4.5	5.0	6.1	7.2	
S.Ed.CD (p=0.05)									
	Treatment		0.1			0.2			
	Duration		0.2			0.4			
	Treatment X Duration		0.4			0.8			

colour was increased significantly with the duration of fermentation. The highest colour score (7.2) was recorded during 24 h and it was followed by 20 h (6.1). The initial colour of the vat solution was considered as colourless and given a grade of one. Interaction of various methods of indigo extraction and different durations of fermentation influenced colour development of the vat solution. The optimum colour grade was 5 and this colour is the end point of fermentation. Among the treatments, the optimum colour (5.3) was observed in 8th hour and retainedupto 12th hour in T_2 (microbial method). The treatment T_3 (hot water treatment) reached the optimum colour at 16th hour of fermentation whereas the other two treatments took about 20 h to develop the optimum colour.

The colour change of water in the steeping vat could be attributed to the action of β-glucosidase from the leaves or bacteria on the *indican* (Perkin and Everest, 1918). Interestingly, Perkin and Everest (1918) noted that during the fermentation stage, *indican* itself does not pass from the leaf into the surrounding liquid. It was recognized that the *indican* is hydrolyzed within the decaying leaf tissue and that the resulting *indoxyl* is leached from the leaves. The tight packing of the fermentation vat was mainly done to exhaust oxygen in the solution. Thus, tight packing prevents the released *indoxyl* from converting prematurely to indigo.

Darrac and Van Schendel (2006) attributed the greenish tinge of steeping vat with light bluish foam to good fermentation and distinguished between a 'good fermentation' and an 'excessive fermentation'. The greenish tinge of the water, which indicates good fermentation, could have resulted from the conversion of *indoxyl* partially or completely into leuco indigo under the predominantly anaerobic conditions of the fermenting vat (Russell and Kaupp, 1969). Using the steeping water colour as an index, it is concluded that for good fermentation, 12 h are recommended for microbial method, 16 h for hot water and 20 h for chemical method. It is interesting to note that excessive fermentation resulted in dark colour. The dark colour of water in the steeping vat may be attributed to presence of *indoxyl* than leuco indigo. Further, *indoxyl* being a highly unstable compound might have entered into unwanted side reactions due to the reactivity of the free *indoxyl* towards a range of compounds leached from the leaves in the steeping stage.

Dye recovery and Indigo content

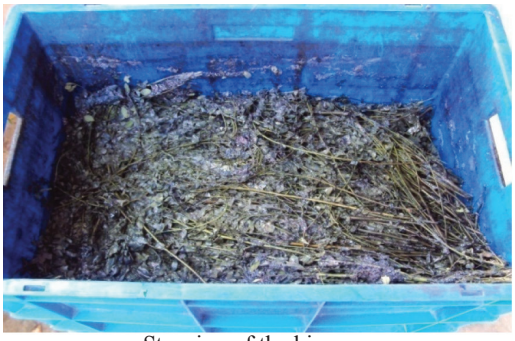
Observations on dye recovery and indigo content revealed significant differences among the treatments (Table 3). Among the four methods of extraction, the highest dye recovery of 1.86 per cent was obtained in T_2 (Microbial method). This was followed by T_3 (Hot water treatment) with 1.70 per cent, T_4 (Chemical method) and T_1 (Conventional method) in the descending order.

Among the various duration of fermentation, the highest dye recovery (1.47 %) was recorded under 20 h of fermentation. The dye recovery was found to be decreased in 24 h of fermentation. The data

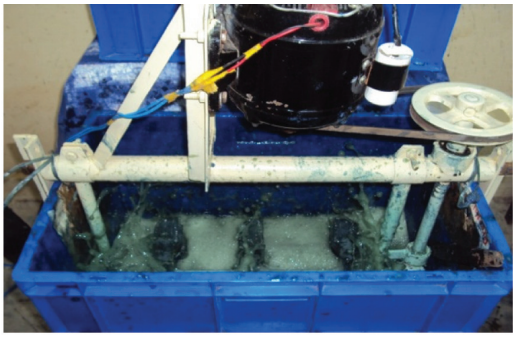
Table 3. Influence of various extraction methods and duration of fermentation on dye recovery (%)

Treatment No.	Method	Duration of fermentation						
		4 h	8 h	12 h	16 h	20 h	24 h	Mean
T_1	Conventional method	0.76	0.79	0.96	1.04	1.18	1.19	1.19
T_2	Microbial method	0.89	1.49	1.83	1.88	1.97	1.99	1.86
T_3	Hot water method	0.78	0.89	1.04	1.64	1.51	1.42	1.70
T_4	Chemical method	0.65	0.75	1.03	1.26	1.21	1.02	1.32
Mean duration of fermentation		0.77	0.98	1.21	1.45	1.47	1.40	
S.Ed.CD (p=0.05)								
	Treatment		0.05			0.12		
	Duration		0.02			0.04		
	Treatment X Duration		0.01			0.02		

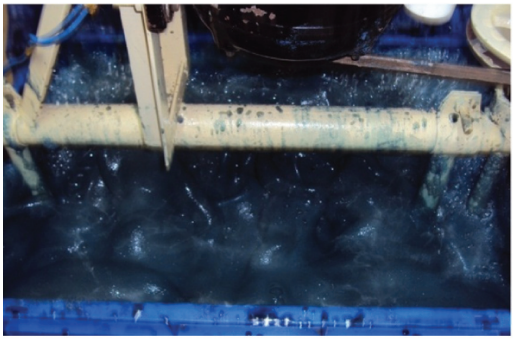
on interaction of the four treatments and the duration of fermentation recorded maximum dye recovery (1.99%) under the microbial method at 24 h of fermentation. However, the other methods showed a decreasing trend in dye recovery after 20 h of fermentation. The reduction in the dye recovery and indigo content observed in the conventional method could also be attributed to failure to sediment indigo



Steeping of the biomass



Oxidation of the soluiton



Dye liquid after oxidation

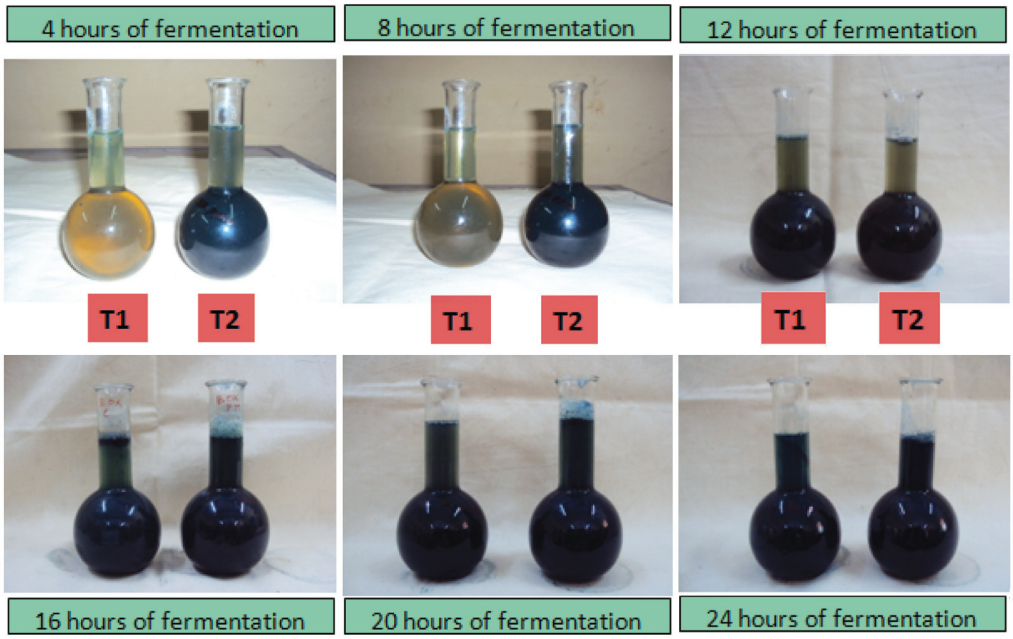
Boiling in copper vessel

Filtering process

Dye slurry and dye powder

Table 4. Influence of various extraction methods and duration of fermentation on indigo content (%)

Treatment No.	Method	Duration of fermentation						
		4 h	8 h	12 h	16 h	20 h	24 h	Mean
T_1	Conventional method	0.45	0.57	0.68	1.37	1.84	1.73	1.69
T_2	Microbial method	0.79	1.96	2.58	2.69	2.78	2.95	2.65
T_3	Hot water method	0.51	1.67	1.93	2.19	2.63	2.41	2.48
T_4	Chemical method	0.36	0.65	0.97	1.26	1.74	1.49	1.88
Mean duration of fermentation		0.53	1.21	1.54	1.88	2.32	2.14	
S.Ed.CD (p=0.05)								
Treatment	0.07	0.16						
	Duration	0.11	0.24					
Treatment X Duration		0.06	0.14					



Extract colour of steeping vat

completely in the oxidation tanks or retention of indoxyl in the leaves and conversion of indoxyl into compounds other than indigo by side reactions with other compounds released from the leaves.

Among the four methods of extraction, the highest Indigo content (2.65 %) was recorded in T_2 (Microbial method) and it was statistically superior to all other treatments. This was followed by T_3 (Hot water treatment) with 2.45 per cent, T_4 (Chemical method) (1.88 %) and T_1 (Conventional method) (1.69 %) in the descending order.

Among the various duration of fermentation, the highest Indigo content (2.32 %) was recorded under 20 h of fermentation. The indigo content was found to be decreased in 24 h of fermentation. The data on interaction recorded maximum indigo content (2.95 %) under microbial method at 24 h of fermentation. After 20 h of fermentation, except microbial method all the other treatments showed decreasing trend in indigo content. This may be due to over fermentation of the solution which does not release the indoxyl and over fermented solution resulted in poor quality with a modified compound. However, due to higher fermentation duration more cell debris and impurities were formed in the dye stuff. Dutta et al. (2017) experienced similar results

and stated that pure indigo yield was more when fermentation duration was extended from 12 to 24 h. The results of the present experiment are in accordance with Shin et al. (2012).

Bechtold et al.(2002) and Darrac and Van Schendel (2006) had recommended addition of a 'small quantity' of an alkali, such as ammonia, sodium carbonate or calcium hydroxide (as slaked lime) after the fermentation stage to improve the yield of indigo. The acceleration of *indoxyl* conversion to indigo at high pH has been described quantitatively by Cotson and Holt (1958). However, when calcium hydroxide was used as alkali, there was a copious production of solid calcium and magnesium carbonates from the dissolved carbon dioxide that had come over from the fermentation vat. These carbonate precipitate helps to carry down the newly formed indigo, but contribute to impurities in the indigo sludge. Hence, it is recommended to wash with dilute HCl, to remove solid impurities that interfere with the dyeing and printing processes. However, as we noticed a raise in pH during oxidation, flocculation agents were not added. This is also reflected in the quality of the samples in all the treatments.

From the above results, it is evident that the active participation of microflora during the fermentation process in breaking down cell lines releasing indican, which gets cleaved of the sugar moiety to indoxyl. The later gets dimerized with oxygen molecule and finally converted to the indigo dye stuff. Looking at the dye recovery and indigo content concurrently at this condition indigo yield was the highest, *i.e*., 1.86 and 2.65 per cent respectively. This infers that 12 h fermentation duration is optimum that favors activity of all factors towards indigo dye production through microbial method.

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