Degradation Of Polyphenol By Bacillus Subtilis Sma Isolated From Effluents Of Tanneries

Dr. Salamun DE, Manesh Shah, Aishwarya Shetty

Abstract: The objective of the present study was to isolate and identify polyphenol degrading microorganism and to optimize the culture conditions for better yield and increased mass production. Waste water samples collected from the effluents of tanneries were filtered and serially diluted. The microbes were grown on nutrient agar medium for 24 hours. The isolated colonies were then transferred onto a mineral salt medium containing various concentration of polyphenol (tannic acid), followed by incubation at 370C for 72 hrs in CO2 incubator. The survival of microorganisms at various increasing concentration of polyphenol (50 ppm to 250 ppm) was performed. The plate which showed highest number of colonies was selected and the isolated colony was subjected to morphological, biochemical and molecular identification. The results showed that the isolate belonged to Bacillus genus and the resulting bacterial strain isolate was found to be Bacillus subtilis and the GenBank accession number was obtained which was MK760577. After identification, the culture conditions required for maximum enzyme production were optimized.

Index Terms: Polyphenols, Tanneries, polyphenol degrading microorganism, GenBank accession number: MK760577

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1. INTRODUCTION

A viable bioremediation technology for hazardous organic pollutants is called Biological degradation. Biological degradation utilizes the metabolic potential of microorganisms to degrade these organic pollutants. The purpose of bioremediation is to provide microbes with nutritive supplements that will encourage them to degrade the organic pollutants and transforming them into harmless metabolites. There are many factors that influence microbes to use these pollutants as a substrate or cometabolize them [1]. Therefore bioremediation is considered as an important mechanism for the removal of organic pollutants from the natural system maintaining environmental stability and compatibility. An important group of pollutant for which efficient biological treatment methods are required are aromatic compounds such as polyphenols [2].

Polyphenols are a class of compounds which consists of hydroxyl groups directly bonded to one or more aromatic rings. The occurrence of polyphenol in a stream is not desirable because when water containing polyphenol is chlorinated, there is production of unpleasant taste and odour in water. The toxicity exhibited by the polyphenol can inhibit the growth rate of marine organisms in water and can affect the ecological balance. Also, the presence of lower concentration of polyphenol in wastewater in irrigated farmland affects the yield of edible crops and presence of higher concentration of polyphenol may lead to crop death. Therefore, the detrimental effects of polyphenols on the environment are limitless, and effective degradation of phenolic contaminants in water is a major concern [3]. Removal of polyphenol from wastewater is currently done by various physicochemical methods such as ion - exchange, ultra-filtration, precipitation /coagulation, osmosis, flotation, electrochemical degradation etc., which are not economic and efficient. These methods also tend to produce other end products which are toxic and require further processing. Therefore, biodegradation of polyphenol is a method of choice and has been studied as an alternative method on the basis of its lower cost and effectiveness [4]. The tannery industry is one of the most polluting industrial sectors. In the process of conversion of animal hides into leather, the tannery industry uses a remarkable amount of chemicals [5]. The process of tanning is a completely wet process that requires significant amounts of water, and about 90% of the used water is generated as effluent [6]. This results in water pollution and creates nauseous conditions in area where tannery industries are located [7].

The effluents generated from tannery industries contains significant amount of wastes, especially tannins. Tannins are a class of naturally occurring complex biomolecules which are water soluble and polyphenol in nature with their molecular weight ranging from 500 to 3000 daltons. Tannins are classified into two types i.e. hydrolysable and condensed tannins. Hydrolysable tannins are subdivided into two groups which are ellagitannins and gallotannins. Gallotannins yield glucose and gallic acid on hydrolysis whereas; ellagitannins yield gallic acid and its derivatives, especially, ellagic acid on hydrolysis. The effluents generated from tannery industries consist of high concentration of tannins. In recent years, studies have been carried out to utilize the metabolic potential of microorganisms for degradation of tannins. The objective of the experiment was to isolate and identify the polyphenol degrading microorganism present in the effluents of tanneries and to evaluate the efficacy of the enzyme produced by the isolate by changing various parameters [8]. The effluents generated from tannery industries contains significant amount of wastes, especially tannins. Tannins are a class of naturally occurring complex biomolecules which are water soluble and polyphenol in nature with their molecular weight ranging from 500 to 3000 daltons. Tannins are classified into two types i.e. hydrolysable and condensed tannins. Hydrolysable tannins are subdivided into two groups which are ellagitannins and gallotannins. Gallotannins yield glucose and gallic acid on hydrolysis whereas; ellagitannins yield gallic acid and its derivatives, especially, ellagic acid on hydrolysis. The effluents generated from tannery industries consist of high concentration of tannins. In recent years, studies have been carried out to utilize the metabolic potential of microorganisms for degradation of tannins. The objective of the experiment was to isolate and identify the polyphenol degrading microorganism present in the effluents of tanneries and to evaluate the efficacy of the enzyme produced by the isolate by changing various parameters [8].

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2 PROCEDURE

2.1 Source of sample

Water sample were collected from the effluents of a tannery industry near a village in Tumkur District.

2.2 Isolation of Polyphenol degrading bacteria

2.2.1 Serial dilution of sample

Water sample collected from the effluents of the tannery industry were filtered and subjected to serial dilutions. A serial dilution is a series of consecutive dilutions to reduce the density of number of cells in a culture. While serially diluting the sample, 1 ml of stock culture was diluted in 9 ml of sterile distilled water producing a 10^{-1} fold solution which was further diluted to 10^{-2}, 10^{-3} and 10^{-4} dilutions.

2.2.2 Isolation of microorganisms in General Purpose Medium

Nutrient Agar is used as a General Purpose Medium. It is used to cultivate and maintain a wide range of non-fastidious microorganisms [9]. It is also used for enumeration of microorganisms i.e. determination of the number of individual viable organisms in a sample; for example in sewage, faeces and dairy products [10].The serially diluted cultures were streaked onto sterile nutrient agar plate and the colonies were allowed to develop by incubating the plates at 37º C for 24 hours.

2.2.3 Isolation of microorganism degrading Polyphenol

A defined mineral salt medium containing different concentration of polyphenol was used for the cultivation and isolation of polyphenol degrading microorganism. Polyphenol was used as the sole source of carbon and all other sources of nutrients were cut down. The polyphenol used in this experiment was tannic acid and the concentrations used were 50 ppm, 100 ppm, 150 ppm and 200 ppm respectively.
Following are the components present in mineral salt medium [11]:

K_2HPO_4 (1.5g/L), KH_2PO_4 (0.5g/L), NaCl (0.5g/L), NH_4NO_3 (1g/L), $MgSO_4.7H_2O$ (g/L), $FeSO_4.7H_2O$ (0.01g/L), $CaCl_2.2H_2O$ (0.01g/L), & NH_4SO_4 (0.5g/L)

The isolated colonies obtained from nutrient agar medium were transferred onto sterile mineral salt media containing different concentrations of polyphenol and the colonies were allowed to develop by incubating the plates at 37ºC for 48 hours. The isolated colonies obtained were subcultured twice onto mineral salt medium containing respective concentration of polyphenol to obtain a pure culture.

2.3 IDENTIFICATION AND CHARACTERIZATION OF THE ISOLATED BACTERIA

The isolate obtained was identified and characterized based on morphological and biochemical tests. Further, complete 16S rRNA sequencing was performed for identification of the isolate. Gram staining was performed for morphological identification followed by colony characterization. Biochemical tests such as Methyl red test, Voges Proskauer test, Indole test and Citrate Utilization test were performed.

2.4 Optimization of different parameters for mass production of enzyme

2.4.1 Carbon Concentration

After the molecular identification of the organism, the isolate was inoculated in MSM broth containing different concentration of polyphenol which was the sole source of carbon. The concentrations of the polyphenol used were 50ppm, 100ppm, 150ppm and 250ppm respectively. It was incubated at 37º C for 48 hours. After incubation, the enzyme activity was calculated by Miller's Method [12].

2.4.2 Nitrogen Source

After the determination of the optimum carbon concentration, the isolate was inoculated in MSM broth with optimum carbon concentration containing different nitrogen source such as Beef extract, Yeast extract and Peptone. One tube was maintained as control i.e. without the addition of external nitrogen source, since MSM broth contains other nitrogen sources like NH_4SO_4 and NH_4NO_3. The inoculated broths were incubated at 37º C for 48 hours. After incubation, the enzyme activity was calculated by Miller's Method.

2.4.3 Temperature

After calculating the C: N ratio, the optimum temperature for the growth of the polyphenol degrading organism was to be determined. For this, the isolate was inoculated in MSM broth containing optimum carbon concentration and nitrogen source and incubated at different temperatures i.e. 28º C, 37º C, 40º C and 50º C respectively for 48 hours. After incubation, the enzyme activity was calculated by Miller's Method.

2.4.4 pH

The isolate was inoculated in MSM broth containing optimum carbon concentration and nitrogen source at different pH i.e. 4, 5.5, 7 and 9 respectively after determining the optimum temperature. The broth was then incubated at optimum temperature for 48 hours. After incubation, the enzyme activity was calculated by Miller's Method.

2.4.5 Static v/s Shaker Condition

The growth and enzyme activity of the isolated organism was then observed in static and shaker conditions. Two set of media were prepared and equal volume of culture were added to each set. One set of flask was kept in static condition and another set was kept in shaker condition at 28º C for 48 hours. After incubation, the growth was determined by taking OD at 540 nm and the enzyme activity was calculated by Miller's Method.

2.5 Production of enzyme in optimized v/s unoptimized conditions

Two set of media were prepared and equal volume of culture were added to each set. One set of the flask were incubated under unoptimized condition whereas, another set of the flask was incubated under optimized condition.

2.6 Application of the enzyme

2.6.1 Removal of Tea Stain

Three small pieces of clean cotton cloth were taken out of which two pieces were treated with tea extract. First piece of cloth was kept as a control. The second piece of cloth was washed with tap water while the third piece of the cloth was treated with crude enzyme and observed [13].

2.6.2 Fruit Juice Clarification

10 ml of juice was mixed with 1 ml of partially purified enzyme, while 1 ml of distilled water was added instead of enzyme in the control tube. This tube was incubated for 15 minutes and the tannin content before and after addition of enzyme was determined by protein precipitation method [14].

2.6.3 Extraction of Coffee Beans

5 g of dried coffee cherries were weighed and were washed with running tap water. These were then immersed in 15 ml of crude enzyme mixture and incubated at 28°C for 7 days in a rotary shaker incubator.

2.6.4 Degradation of Coffee Cherry Husks

5 g of dried coffee cherry husks were weighed and were immersed in 15 ml of crude enzyme mixture. This was then incubated at room temperature for 2 weeks.

TESTS	RESULTS
Indole test	Negative
Methyl red test	Positive
Voges Proskauer test	Negative
Citrate Test	Positive

Table 1 : Colony Characters of the bacterial strain

3. RESULTS AND DISCUSSION

3.1 Isolation of polyphenol degrading organism

The sample collected from the effluents of tannery industry which were subjected to serial dilutions and streaked onto nutrient agar plates (Figure- 1). The plates showed growth of the colonies which were then transferred onto Mineral Salt Medium agar (Figure- 2).

***Figure 1**: Isolation of bacteria from diluted sample on Nutrient Agar*

***Figure 2 :** Isolation of Polyphenol degraders on MSM containing Polyphenol as sole source of carbon*

The organism which was able to utilize polyphenol as a sole source of carbon developed on Mineral Salt Medium agar plates. These isolated colonies were further subcultured to obtain a pure strain.

3.2 Identification and characterization of the isolated bacteria

Gram staining was performed for morphological identification of the organism followed by colony characterization. For biochemical characterization, MR-VP test, Indole production test and Citrate utilization test were performed (Table-1 & 2).

CHARACTERISTICS	OBSERVATION
Shape	Circular
Size	2mm
Colour	White
Elevation	Flat
Margin	Entire
Consistency	Butyrous
Opacity	Opaque
GRAM Character	GRAM positive
Morphology	Rod shaped

Table 2 Biochemical characteristics

3.3. Molecular identification

The isolated bacterial strain was subjected to partial 16S rRNA sequencing and the obtained 1203bp 16S rRNA nucleotide sequence was compared with the available 16S ribosomal sequences in the NCBI database using BLASTn. The polyphenol degrading bacteria has been enrolled into a cluster containing Bacillus sp. and was found to be closely related to Bacillus subtilis (Figure-3). The nucleotide sequence was submitted, was provided with a Gen bank accession number: MK760577.

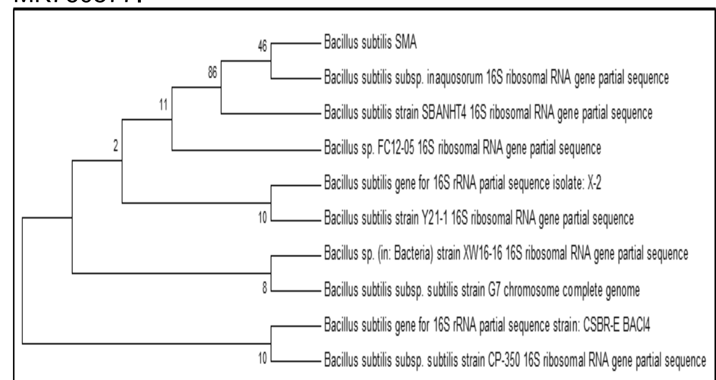


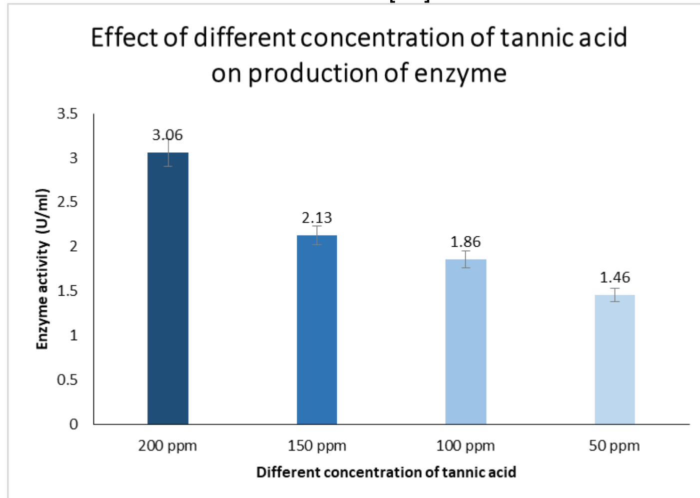
***Figure 3** : Phylogenetic tree*

3.4. Optimization of different parameters for mass production of enzyme

3.4.1 Carbon Concentration

The isolate was inoculated in MSM broth containing different concentration of polyphenol which was the sole source of carbon. The concentrations of the polyphenol used were 50 ppm, 100 ppm, 150 ppm and 250 ppm respectively. It was observed that the enzyme activity was highest in the medium containing 200ppm of polyphenol (3.06 U/ml) followed by 150 ppm (2.13 U/ml) and 100 ppm (1.86 U/ml). Least enzyme

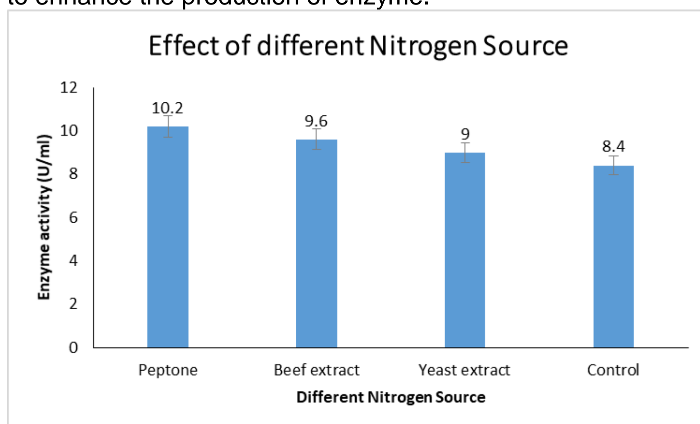
activity was observed in the medium containing 50 ppm of polyphenol (1.46 U/ml) (Graph-1). Our findings were in agreement with the previous literature which showed that A. niger (Lekha & Lonsane, 1994), Aspergilus oryzae (Lekha & Lonsane, 1997) and Paecilomyces variotii (Battestin & Macedo, 2007) showed increase in tannase production with increase in amount of the inducer [15].



Graph 1 : *Effect of different concentration of tannic acid on enzyme activity of Bacillus subtilis SMA*

3.4.2 Nitrogen Source

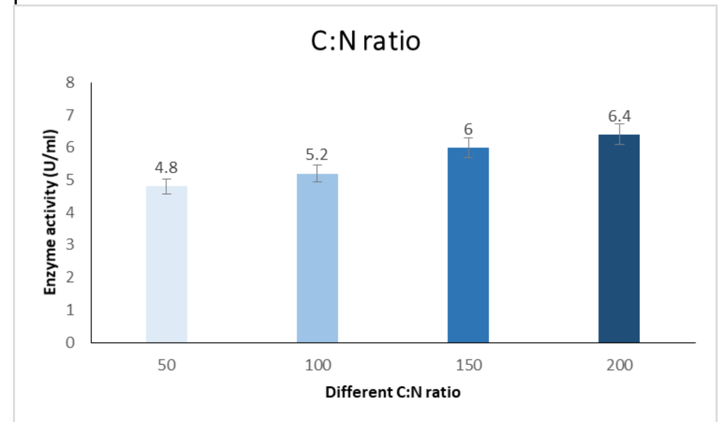
After determination of the optimum concentration of carbon, the isolate was inoculated in MSM broth containing different nitrogen source such as Beef extract, Yeast extract and Peptone and one tube was maintained as a control. It was observed that medium containing 0.3% Peptone showed highest enzyme activity (10.2 U/ml) followed by Beef extract (9.6 U/ml) and Yeast extract (9 U/ml). The control tube showed least enzyme activity (8.4 U/ml) (Graph-2). These results indicate that organic sources are more effective for production of enzyme than inorganic source. Our observation for the use of organic nitrogen source was first of its kind and can be used to enhance the production of enzyme.



Graph 2 *: Effect of different Nitrogen source on enzyme activity of Bacillus subtilis SMA*

3.4.3. C: N Ratio

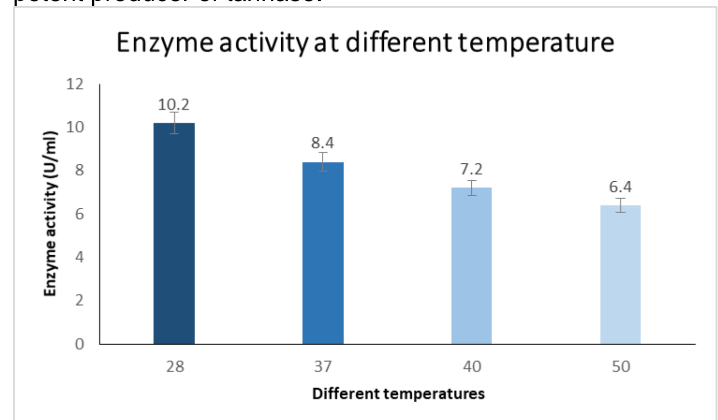
The variable concentration of tannic acid (50 ppm, 100 ppm, 150 ppm and 200 ppm) and fixed concentration of peptone (0.3%) was added to the culture medium to determine the optimum Carbon: Nitrogen ratio. Highest enzyme activity was observed with C: N of 200 ppm of tannic acid: 0.3% of peptone (6.4 U/ml) followed by 150 ppm: 0.3% (6 U/ml), 100 ppm: 0.3% (5.2 U/ml) and 50 ppm: 0.3% (4.8 U/ml) respectively (Graph-3). This is the first report to study the different C: N ratio used for degradation of tannic acid by Bacillus subtilis SMA, and can be used for potential increase of enzyme production.



Graph 3 : *Effect of different C: N ratio on enzyme activity of Bacillus subtilis SMA*

3.4.4 Temperature

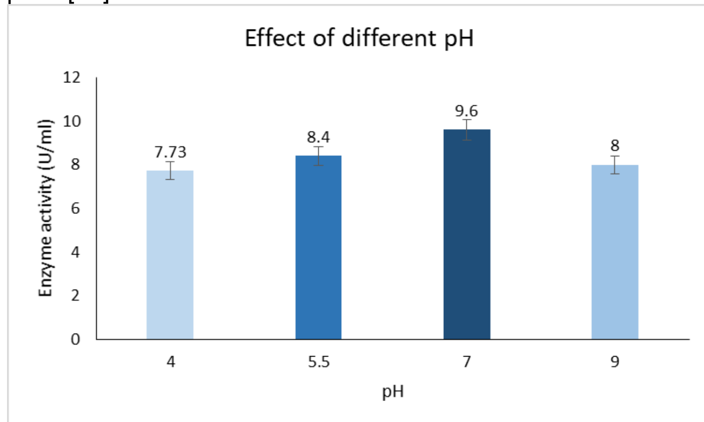
Temperature is one of the major factors influencing enzyme activity. At optimum temperature, maximum enzyme activity is achieved. The identified isolate was inoculated in MSM broth and incubated at different temperatures i.e. 28º C, 37º C, 40º C and 50º C respectively for 48 hours to determine the optimum temperature for enzyme activity. Maximum enzyme activity was observed at 28º C (10.2 U/ml) followed by 37º C (8.4 U/ml) and 40º C (7.2 U/ml). Least enzyme activity was observed at 50º C (6.4 U/ml) (Graph-4). In the study by Aftab et al the variable strain of Bacillus subtilis, was able to produce maximum titre of tannase at 41ºC with 0.34 U/ml, which was very low compared to our strain. Previous literature studies have also indicated that the best temperature ranges from 28-34ºC for Paecilomyces variotii, Rhizopus oryzae and Aspergillus niger PKL 104 on tannase productions [16]. Therefore, it can be suggested that Bacillus subtilis SMA is a potent producer of tannase.



Graph 4 *: Effect of different temperature on enzyme activity of Bacillus subtilis SMA*

3.4.5 pH

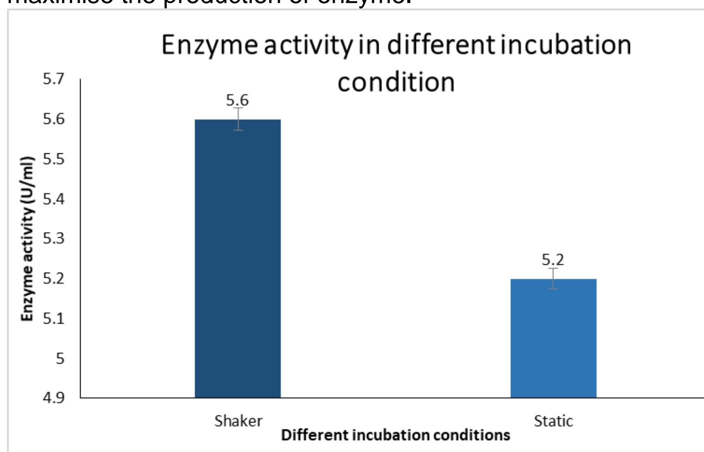
The pH of the media is one of the major factors influencing the production of enzyme. Therefore the pH of the medium was analysed for the optimum growth of the organism and its enzyme production. The isolate was inoculated in MSM broth of different pH i.e. 4, 5.5, 7 and 9 respectively after determining the optimum temperature. Maximum enzyme activity was observed at pH 7 (9.6 U/ml) followed by pH 5.5 (8.4 U/ml) and pH 9 (8 U/ml) which was almost similar. Least enzyme activity was observed at pH 4 (7.73 U/ml) (Graph-5). Literature reports have shown different strain of Bacillus subtilis was found to exhibit maximum enzyme production at pH 5 [16].



Graph 5 : *Effect of different pH on enzyme activity of Bacillus subtilis SMA*

3.4.6 Static v/s Shaker condition

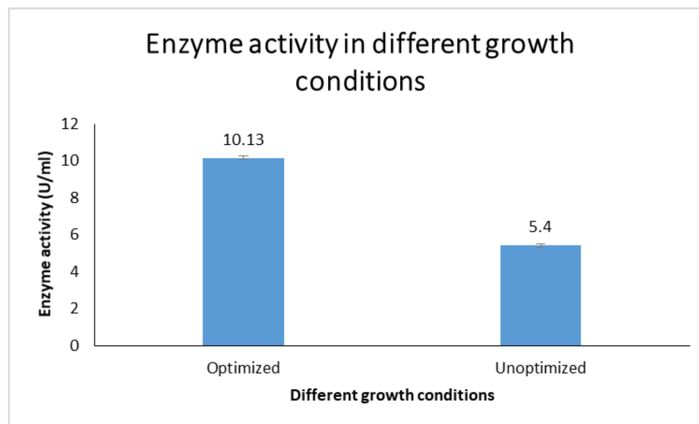
The growth and enzyme activity of the isolated organism was observed in static and shaker conditions under optimized conditions. It was observed that enzyme activity was higher in shaker condition (5.6 U/ml) than static condition (5.2 U/ml) (Graph-6). This may be because the bacteria is aerobic in nature. Shaker conditions ensures aeration and nutrient availability as well as it avoids bacterial settlement in the bottom of the flask. Also, shaking prevents bacterial clumps or biofilm formation, ensuring prolipheric bacterial reproduction. The present study on Bacillus subtilis SMA under different incubation conditions was first to report that shaker conditions maximise the production of enzyme.



Graph 6 : *Effect of different incubation condition on enzyme activity of Bacillus subtilis SMA*

3.4.7 Production of enzyme in optimized v/s unoptimized conditions

The production of enzyme by the isolated organism in optimized and unoptimized conditions was determined. The inoculated broth kept under optimized condition showed higher enzyme activity (10.13 U/ml) than unoptimized condition (5.46 U/ml) (Graph-7). Activity of tannase was increased nearly by two fold when optimized conditions were provided.



Graph-7: *Effect of optimized and unoptimized growth conditions on enzyme activity of Bacillus subtilis SMA*

4. APPLICATIONS OF ENZYME

4.1 Removal of Tea Stain

A white cotton cloth which was nottreated with tea extract was used as control (Figure-3.a). The cloth that was stained with tea extract when washed under running tap water did not remove the stain (Figure-3.b). The third piece of cloth that was stained with tea extract was subjected to enzyme treatment resulting in destaining of the cloth (Figure-3.c). Our finding was in line with that of Aida M. Farag et. al, showed that the application of A.nomius GWA5 tannase in removed tannin stains of tea.

Figure 3.a: *Cloth used as control*

Figure 3.b : *Tea stained cloth*

Figure 3*.c Tea stained cloth treated with crude enzyme*

4.2 Fruit Juice Clarification

The tannic acid content of the fruit juice was determined by using protein precipitation method and was found to be 1060 µg/ml. After treating the juice with the crude enzyme mixture, the tannin content of the juice was decreased down to more than three fold i.e. after treatment with the crude enzyme mixture, the tannin content in the fruit juice was found to be 370 µg/ml (Figure-4.a and Figure-4.b). Determination of tannin content was also done at different time intervals i.e. at 0.5hrs, 16 hrs, 20 hrs and 24 hrs. It was observed that there was gradual decrease in tannin content with increase in time. After 30 minutes the tannin content was reduced from 1060µg/ml to 370 µg/ml. After 16 hours it reduced further to 260 µg/ml and after 20 hours the tannin content was reduced to 240 µg/ml and then remained the same.

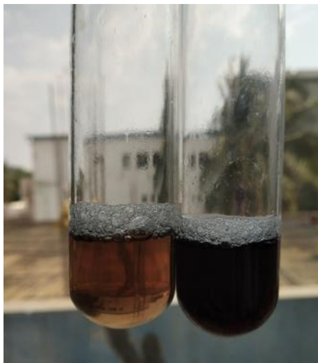


Figure 4.a *(left) : Juice sample treated with enzyme*
Figure 4.b *(right) : Juice sample untreated*

4.3 Extraction of Coffee Beans

5g of dried coffee cherries were incubated in crude enzyme for 7 days and were found to have become soft, which were then easily peeled off to get the coffee beans. This method for extraction of coffee beans from the coffee cherries will lower the cost and the lengthy process of pulping (Figure-5).

Figure 5 : *Coffee extracted from coffee cherries*

4.4 Degradation of Coffee Cherry Husks

5g of dried coffee cherry husks were incubated with the crude enzyme for 15 days, after the incubation period the hard dried husks were converted to soft slurry. This is because of the action of enzyme upon the dried husks which resulted in degradation of the dried coffee cherry husks. The degradation of coffee cherry husks usually takes around 35-40 days, however, by the application of this enzyme the degradation can be brought about in less than half the time required by its natural degradation (Figure-6).

Figure 6 : *Soft slurry of coffee cherry husks*

5. CONCLUSION

The present study aimed to isolate and identify a bacterial strain that degrades polyphenol i.e. tannic acid by producing the enzyme tannase. Optimization of culture conditions were performed for maximum degradation of polyphenol. The effluent samples were collected from a leather industry near Tumkur district. The sample was subjected to serial dilutions and inoculated on Nutrient Agar plate. For isolation of polyphenol degrading organism, the isolated colonies obtained from nutrient agar were transferred to Mineral Salt Media containing polyphenol and subcultured twice to obtain a pure strain. The isolated organism from MSM media was considered as polyphenol degrading bacteriaMorphology, colony assay and biochemical characterization were performed for the selected organism which showed that the selected strain was Gram positive rods which utilized citrate as a sole source of carbon and produces stable acids by utilizing glucose by mixed acid pathway. Further 16S rRNA sequencing was carried out to identify the selected organism. Molecular

identification showed that the selected bacterial strain was a new strain of Bacillus subtilis which was named as Bacillus subtilis SMA and the Gen bank accession number was MK760577. After morphological and molecular identification, optimization of culture conditions was carried out for maximum enzyme production by Bacillus subtilis SMA to degrade tannic acid as a source of polyphenol in this study. Different concentration of tannic acid was used as a substrate to observe the production of enzyme by Bacillus subtilis SMA. Increased enzyme activity was observed at substrate concentration of 200 ppm (3.06 U/ml). It was observed that enzyme production increased with increase in substrate concentration. Different nitrogen source was used for optimizing the culture condition, of which peptone (10.2 U/ml) showed the highest enzyme activity. The isolated strain Bacillus subtilis SMA was observed for its enzyme production at different C: N ratio. It was observed that highest enzyme activity was observed at C:N ratio of 200 ppm : 0.3 % (6.4 U/ml). The strain Bacillus subtilis SMA was analysed for its ability to degrade polyphenol at different incubation temperature i.e. 28°C, 37°C, 40°C and 50°C. Maximum tannase production was observed at 28°C showing tannase activity of 10.2 U/ml. pH of the media plays an important role in inducing the production of enzyme. The isolate Bacillus subtilis SMA was analysed for the enzyme production at various pH – 4, 5.5, 7 and 9; and maximum tannase production was observed at pH 7 (9.6 U/ml). The strain Bacillus subtilis SMA was analysed for its ability to degrade tannin rich coffee cherry husks, destaining of tannin stains of tea, for juice clarification and for removal of coffee beans from coffee cherries. The tannase secreted by the bacterium Bacillus subtilis SMA efficiently destained the cotton cloth with 5 minutes of enzymatic treatment which showed the potential of the tannase for its commercial application. Coffee cherries when dipped in crude enzyme showed that the covering turned soft and squishy after incubation of a week, this soft covering was then easily removed to get the coffee beans. This method can be used in place of costly procedure of pulping which requires high amount of energy and labour and also produces large amount of waste. Coffee cherry husks were degraded within 2 weeks of incubation in tannase secreted by Bacillus subtilis SMA. This process of biodegradation of coffee cherry husks is environment friendly and can degrade husks quicker than the traditional physicochemical process. Tannase extracted from Bacillus subtilis SMA was also used for juice clarification, tannin content in fruit juice contributes to the haziness and bitterness of the juice, the use of tannase decreased the tannin content in the juice sample by almost 3 folds also it resulted in clear juice, reducing the haziness and also resulted in reduction of bitterness.

6. ACKNOWLEDGEMENT

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