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# Extraction of tomato carotenoids, mediated by crude bacterial cellulase

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**Abstract :** *Cellulose is the most abundant polymers on earth. It is degraded by the enzyme cellulase .This enzyme is produced by the several microorganisms. Carotenoids are the most widespread naturally occurring yellow to orange pigment which is used as a colouring agent. The synthetic colouring agent expose immense health hazards. Thus, the main aim of the study was to isolate efficient cellulase producing bacterial strains from soil. And use to these bacterial strains to produce maximum crude cellulase by optimizing various parameters. Further the crude enzymes were used for the extraction of carotenoids from tomato. It was seen by thin layer chromatography that extraction mediated by cellulase enhanced the process.*

***Keywords***: *Crude enzymes, Tomato homogenate, cellulase, cellulose.*

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## Introduction :

Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products which are of great value in day to day life. It is commonly degraded by an enzyme called cellulase. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β exoglucanase, and β-glucosidase or cellobiase. Endoglucanase is responsible for the random cleavage of the glycosidic bonds along a cellulose chain. Exoglucanase is necessary for the cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose. Only the synergy of the three enzymes makes the complete cellulose hydrolysis. Extensive basic and applied research on cellulases revealed the commercial significance and industrial applicability of this enzyme (Bajpai, 1999).

Carotenoids are the most widespread naturally occurring yellow, orange and red pigments used as food colourant. Carotenoids also play a significant role in human health as precursors of vitamin A, scavengers of active oxygen, enhancers of in vitro antibody production, anticancer agents and so on.

Recently, carotenoids have attracted greater attention due to the beneficial role on human health. Carotenoid can inhibit various types of cancer and it enhanced the immune response beside carotenoids, increasing their antioxidant potential (Martinez-Valvarde et al., 2002). Tomatoes and their derived products (tomato juice, ketchup, tomato paste) are essential food, used worldwide in the human diet, and recommended for disease prevention due to the high content of lycopene, beside β- carotene (Zuorro et al., 2011).

The classical method to extract carotenoid and to evaluate their concentration in tomatoes is the "organic solvent procedure", using toxic solvents like petroleum, benzene, ethyl ether and methanol. The extraction of lycopene by conventional food – grade organic solvents (ethanol) is significantly low, due to its strong insertion in chromoplastic wall structure and the *in viv*o activity of carotenoids is affected.

The cellulase enzyme has successfully been used to facilitate the release of phenolic compound (Landbo and Meyer, 2001) or some types of carotenoids from different plant materials. This enzyme facilitates carotenoids extraction from tomato paste or pulp, obtaining higher yields of recovery of lycopene (Zuorro and Lavecchia, 2010).

**Materials and Methods :**

Soil sample from different places were collected in sterilized polythene bags and then they were further processed within 24 hr of procurement.

0.1g of soil sample was serially diluted in 10 ml of sterilized normal saline upto $10^{-6}$. Nutrient agar media was prepared, autoclaved and poured into sterilized petri plates under sterile condition. Plates were left for solidification and were inoculated with the dilution of $10^{-4}$, $10^{-5}$ and $10^{-6}$ by spread plate method. Then the plates were incubated at 37°C for 24 hours.

Out of the different colonies obtained on NA plates, five colonies (CB1, CB2, CB3, CB4 and CB5) were selected based on the cultural characteristics and were streaked on Nutrient agar slants and maintained for further use with periodic subculturing.

The isolates streaked on NA slants were grown on carboxymethyl cellulose (CMC) agar media (pH 7.0).The plates were incubated at 37°C for 48 hr to allow the secretion of cellulase and degradation of cellulose present in the form of CMC. After incubation the isolates were qualitatively and quantitatively tested for cellulase activity.

For qualitative test (clear zone) CMC agar media plate with isolated colonies were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1M NaCl (Apun *et al*, 2000). The strains showing a clear zone due to utilization of CMC was selected as potential cellulolytic strains for further study (Beguin and Anbert ,1994).

For further quantitative estimation (amount of glucose released) of cellulase activity, each of the three isolates was incubated in CMC broth for 24 to 48 h separately. It was then centrifuged at 5000 rpm for 15 minutes. The supernatant was collected which act as crude enzyme and stored at 4°C for further use. 0.5 ml of crude enzyme was taken in test tube and then 1ml of 0.05M sodium citrate buffer (Mandels and Weber, 1969) was added, further pinch of CMC was added to it. The test tube with sample was incubated at 50°C for one hour in incubator. At the end of incubation period 3ml of DNS reagent was added and then boiled in water bath for 5 minute. Then, the tube was cooled in ice cold water. The amount of reducing sugar released by the hydrolysis of CMC was measured at 540nm by DNS (Miller 1959) using glucose a standard.

Most efficient cellulolytic bacterial strain was maintained by periodic sub culturing and kept at 4°C.

The best bacterial isolate with maximum cellulase production was further identified on the basis of cultural characteristics and morphological characteristics.

The isolated colonies were identified on the basis of colour, margin, texture, and elevation of colony on nutrient agar media.

Culture was characterized morphologically by Gram's staining (Han's Gram) and colony characteristics like colour, margin, texture, and elevation.

**pH:** pH is an important factor affecting the microbial growth as well as enzyme production. Therefore, different pH values (4.5, 5.5, 6.5, 7, and 7.5) were taken to study the effect on cellulase production.

Temperature is an important factor in a bioprocess for the production of extra-cellular enzyme. For the selection of optimum temperature for cellulase production, the isolated culture was incubated at different temperature (26°C, 32°C, 37°C, 45°C, and 55°C) for 48 h.

The effect of incubation time on cellulase activity was observed by incubating the isolate at 37°C for different time interval (24, 48, 72, 96,120, 144, 168, 192, 216 and 240) h respectively.

Various nitrogen sources like ammonium chloride, ammonium sulphate, ammonium nitrate, yeast extract, peptone were used and examined for their effect on enzyme production by replacing tryptone in the production medium.

The effect of different substrates as carbon source like rice bran, sugarcane bagasse, saw dust, coconut husk, pineapple peel, grasses were tested for their effect on enzyme activity by isolated culture (Mandels and Weber, 1969).

Fresh tomato was used for Carotenoid extraction. Firstly, the tomato pulp was extracted and finely homogenized. 1ml of homogenate was then treated with 1ml of crude cellulose enzyme obtained from selected bacterial strain (Zuorro et.al 2011) grown on pineapple and peptone as its nitrogen source. The homogenates was then incubated for 2 h. The homogenate was then placed in air containers.

The homogenate was then subjected to Thin layer chromatography using silica gel. Hexane, acetone and ethanol in the ratio 50:25:25 (v/v) were used as solvent for separation. (Jhojhima and Ogura , 1983)

**Results and Discussion :**

The five isolates (CB1, CB2, CB3, CB4 and CB5) obtained from soil sample on nutrient agar media. These isolates were maintained on CMC for screening.

Out of five isolates obtained on NA media plates only three were able to grow on CMC media (CB1, CB2, and CB4). The largest clear zone was seen in CB4 isolate in congo red test. Since the sole carbon source in CMC media was carboxy methyl cellulose, the clear zone in the medium indicated cellulose degradation by the isolates. The clear zone around the colonies shows the production of cellulase enzyme by the bacterial culture which degraded the cellulose present in medium.

The potential isolate (CB4) was identified on the basis of cultural and morphological characteristics.

The bacterial isolate showed the following characteristics on the agar media and under microscope at 40X as shown in Table1.

**Table 1. Cultural and morphological characteristics of bacterial isolate**

| Isolate | Colour | Texture | Margin | Elevation | Gram's reaction | Shape |
|---|---|---|---|---|---|---|
| CB4 | Creamy | Slimy | irregular | Flat | Gram positive | *Streptococcus* |

Isolated bacterial isolated was allowed to grow in media of different pH ranging from 4.5, 5.5, 6.5, 7, 7.5. (Lugani et al., 2015). Maximum enzyme production was observed in medium of pH 5.5 (Fig. 1).

Isolated bacterial isolated was allowed to grow in media of different temperature ranging from 26°C, 35°C, 37,45°C,55°C. Maximum enzyme production was observed at 37°C. (Fig. 2).

Production of extracellular cellulase has been shown to be sensitive to different nitrogen sources. The effect of nitrogen sources was studied in the growth medium, where tryptone was replaced by different nitrogen sources such as $NH_4Cl$, $NaNO_3$, yeast extract and peptone. Among the various nitrogen sources tested, peptone was found to be best nitrogen sources for cellulase production for the isolated bacterial culture (Fig.3).

Enzyme was recorded at different incubation periods i.e., 24 h to 240 h. As seen from graph shown below there was a sudden increase in enzyme production and maximum was obtained at 120 h of incubation and decreased above this due to depletion of nutrients or accumulation of other by products (Fig.4)

Different natural carbon sources such as coconut, pineapple, saw dust, sugarcane, rice bran was used instead of CMC to estimate their effect on cellulase production. The maximum production was seen in case of pineapple peel followed by coconut and rice bran as shown in Fig.5 (Mandels and Weber, 1969)

To analyse extracted Tomato Carotenoids Thin-Layer Chromatography was performed. The Rf value of the different coloured spots was calculated. It was seen that in case of tomato homogenate devoid of crude enzyme only a single spot whereas in case of tomato homogenate treated with crude enzymes, three distinct spots were obtained. The Rf values and the probable identification of carotenoids based on colour is shown in Table 2. The results obtained by Thin-Layer chromatography technique demonstrate the possibility to enhance the extraction of carotenoids from tomato tissue by using cell wall degrading enzymes such as cellulases.

**Table 2. Chromatographic separation and probable identification of tomato carotenoids**

| S. No. | Homogenate | No. of spots obtained | Colour of the spot | Rf value | Probable identification of carotenoids based on colour |
|---|---|---|---|---|---|
| 1. | Tomato (control) | 1 | orange-red | 0.43 | lycopene |
| 2. | Tomato + crude enzyme | 3 | orange | 0.81 | carotene |
| | | | Yellow-orange | 0.65 | lycopene |
| | | | Orange- red | 0.44 | lycopene |

## Conclusion :

Out of the five isolates, two were screened for cellulase production using CMC media, of which one was selected for optimization on the basis of Congo red test and enzyme assay. The most optimal condition for cellulase production from CB4 was at pH 4.5, 37°C temperature and incubation period of 120 h. The present study proved that peptone is a good nitrogen sources for maximum production of cellulase enzyme. Natural substrate such as pineapple peel, coconut husk, saw dust, rice bran etc. are good carbon sources out of these pineapple was found to be better. The crude enzyme produced from CB4 under the procured optimized condition was used for the release of carotenoid from tomato homogenate. The results obtained in thin chromatography technique demonstrate the possibility to enhance the extraction of carotenoid from tomato tissue by using cell wall degrading enzymes such as cellulases. Thus the crude enzyme produced from CB4 under the optimized condition enhanced the release of carotenoid from tomato homogenate.

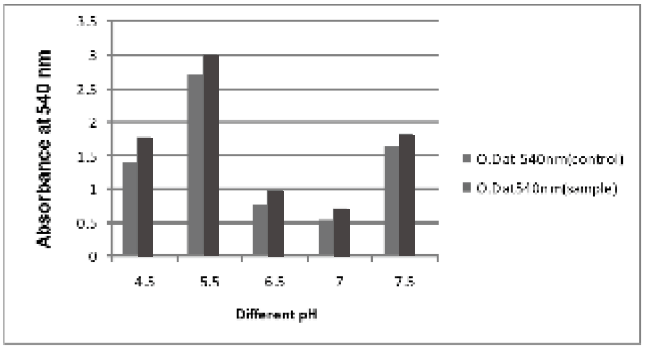


**Fig. 1. Effect of pH on growth**

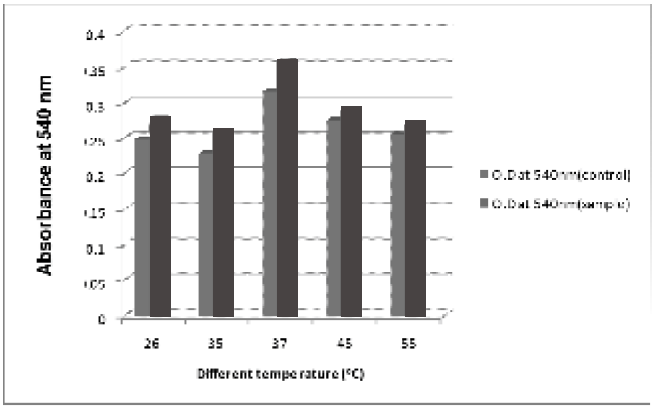


**Fig. 2. Effect of temperature on growth**

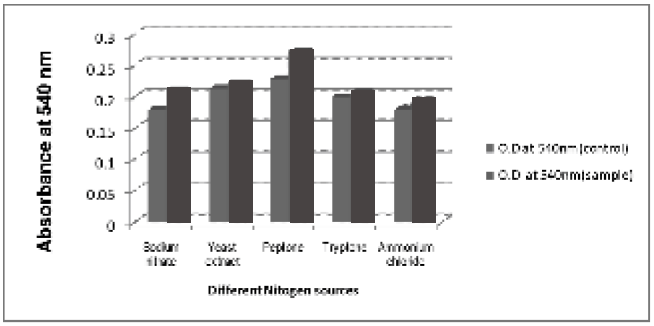


**Fig. 3. Effects of nitrogen substrate on growth**

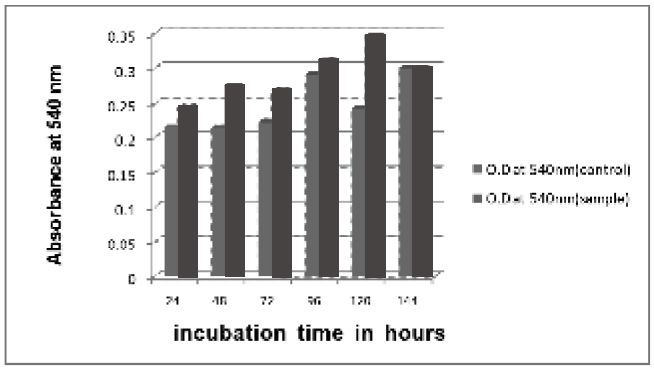


**Fig. 4. Cellulase activity of control and sample at different incubation period**

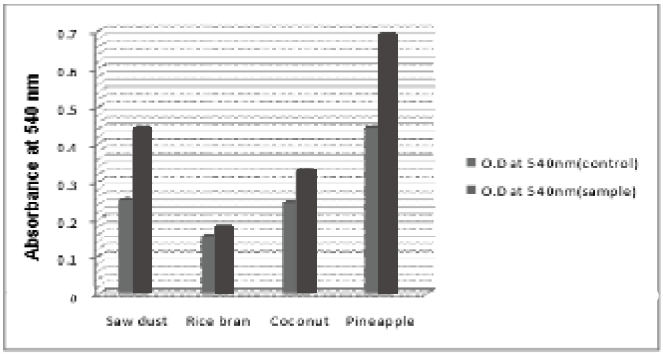


**Fig. 5. Effect of different carbon source on cellulase production**

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