RESEARCH

24(84), October 1, 2014

ISSN 2278–5469 EISSN 2278–5450

Discovery

# Kinetic Determining Innovations of Carboxymethyl Cellulase Enzyme Isolated from *Trichophyton terrestre* in Carboxymethyl Cellulose Solution

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**Publication History**

Received: 08 August 2014

Accepted: 19 September 2014

Published: 1 October 2014

**Citation**

Mansoor A Lone, Sanjay Sahay, Ravinder Rana, Manzoor A Rather, Farroq A Dar, Firdous A Malla, Muzaffar A Reshi. Kinetic Determining Innovations of Carboxymethyl Cellulase Enzyme Isolated from *Trichophyton terrestre* in Carboxymethyl Cellulose Solution. *Discovery*, 2014, 24(84), 110-117



**ABSTRACT**

The techniques employed for bioconversion of cellulosic biomass to simple sugars by carboxymethyl cellulase have a great industrial importance. Cold-adapted microorganisms serve as potential resource of cold-active carboxymethyl cellulose.In this study, *Trichophyton terrestre,* a rare fungal species in Indian soils, isolated from the rhizosphere of *Juglansregia* L. during winter season was

used for the production of carboxymethyl cellulase in carboxymethyl cellulose solution by DNS method at various range of temperatures, using Lineweaver-Burk plot which offers a practical graphical method for the analysis of Michealis-Menten equation, for finding enzyme kinetics, such as Km and Vmax. The enzyme kinetic parameters like maximum activity (Vmax), Km and turnover number were recorded at various concentrations of CMC and different temperatures 4˚ C and 50 ˚ C. The enzyme was found to be tolerant and stable at wide range of temperatures with substantial residual cold-activity which enables this fungal species to survive in extreme environmental conditions of northern India. Such property of carboxymethyl cellulase enzyme has extensive range of applications at industrial scale.

**Key words:** Carboxymethyl cellulase, *Trichophytonterrestre,* CMC, cold-activity.

## 1. INTRODUCTION

Human kind has been benefited enormously from the study of microbes since their discovery in the 17$^{th}$ century. However, such benefits have come from the study of just a miniscule fraction of the millions of the species of microbes in the environment (Anne and Ann, 2007). The enormous returns have been particularly derived from those microbes which are able to live in the severe environmental conditions like low or high temperature, extremes of pH, high salt concentrations, nutrient deficient soils, etc. The ability of surviving in such environmental conditions might be related with enzyme stability. Sometimes these microbes are called as the nature's 'master chemists' because an endless variety of chemical compounds are derived from their community. They produce the novel and stable enzymes which function under extreme conditions comparable to those prevailing in various industrial processes. Intense environments always provide a unique resource of microorganisms and novel biocatalysts. *Trichopyton terrestre* was isolated from the rhizosphere of *Juglans regia* L. in the Kashmir valley, India, which has its unique environment throughout the world. Cellulose is the structural polysaccharide in plants and the most abundant biomass on earth. It is composed of β-1, 4-linked glucose units which contains both highly crystalline and amorphous regions (Zhang and Lynd, 2004). Researchers have been stimulated to hydrolyze cellulose to soluble sugars by microorganisms for industrial processes owing to great potentiality of this abundant natural product as an alternative energy source (Coughlan, 1990). Cellulosic materials are converted into soluble sugars by many methods like acid hydrolysis, pyrolysis and by employing enzyme celluloses (Cooney et al., 1978). The acid hydrolysis of cellulosic materials is cheaper than the cellulase hydrolysis, but the former often requires high temperature, pressure and leads to the accumulation of repulsive by-products (Fennington et al., 1982). On the other hand, enzymatic hydrolysis does not have these tribulations. Mostly bioconversion of cellulose by enzymes is employed in the industrial processes to synthesize commercially important products. Cellulase refers to the family of enzymes that work to hydrolyze cellulose. There are numerous microorganisms including both bacteria and fungi which have been found to produce a variety of cellulases for the degradation of cellulose (Miranda et al., 2011), but only a restricted group is capable to produce a sufficient amount of cell-free cellulase, which is proficient in completely hydrolyzing the crystalline cellulose *in vitro*. For the degradation of cellulose, fungi utilize the hydrolytic enzymes viz., exo-cellulase, endo-cellulase, cellobiohydrolase, endoglucanase and P-glycosidase (Bhat, 2000), of which endoglucanases has special significance as it catalyze hydrolysis of internal bonds of cellulose i.e. β- 1,4- glucosidic bonds. Continuing research on *Trichophyton terrestre* indicated that the microorganism has a complete set of cellulase enzymes required for the breakdown of cellulose to glucose (Reese et al., 1950). Cellulase enzymes including endoglucanases indicate the potentiality of successful conversions of waste cellulose into foods for our growing population, thus the enzyme exploitation subject demands the intense research even at molecular level (Eveleigh, 1985).

## 2. MATERIALS AND METHODS

### Isolation and identification

Soil samples were collected from 20 cm deep the rhizosphere of *Juglans regia L. randomly in aseptic manner i*n the Northern regions of India (Kashmir valley) during three different seasons-rainy, spring and winter. Fungal colonies were isolated by serial dilution method to get more manageable results (Aneja, 2005). 1g of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to $10^{-6}$. Czapek-Dox agar medium was used as culturing media with composition of (g/l); sucrose- 30, $NaNO_3$- 3, $K_2HPO_4$- 1, $MgSO_4$- 0.5, KCl- 0.5, $FeSO_4$- 0.01, agar agar- 15, pH of the medium was adjusted to 7.3. After autoclaving at 121°C and 15 lbs pressure, 20 ml of sterile medium was transferred to sterile petri-plates (Chloramphenicol 250mg/100ml was also added to check the bacterial growth). 0.1 ml of soil suspension was spread with the help of spreader and incubated at 28˚C for 7 days. The fungal cultures grown on the medium were transferred on to the Potato Dextrose Agar (Hi Media) medium and pH was maintained at 5.6 for further studies. Identification of fungi was done based on cultural, morphological and microscopic characters.

**Cellulase production**

Among the identified cultures, *Trichophyton terrestre* was selected. A volume of 100 ml of Czapek-Dox broth medium amended with 1% cellulose was distributed into separate 250 ml Erlenmeyer conical flask. The pH of the medium was adjusted to 5. After autoclaving at 121˚C and 15 lb pressure, the fungal spore suspension was inoculated into the conical flask with the inoculum concentration of $1x10^6$ spores $mL^{-1}$. The flask was incubated at 32°C on a rotary shaker at 120 rpm for 7 days (Lone et al., 2012). After incubation, the content of the flask was passed through Whatman filter paper No.1 to separate the mycelial mat from culture filtrate. The filtrate was used for the estimation of extracellular protein content and total activity of endoglucanases.

**Protein estimation**

Protein content of the supernatant secreted by fungus was estimated according to Lowry et al., (1951). The experimental data was collected and glucose standard curve (Figure 1) was used as a standard (Kondo et al., 1994).

**Kinetics of enzyme endoglucanases**

The enzyme endoglucanase in the crude extract was assayed with increasing concentrations of carboxymethyl cellulose (0.16-0.83mg/0.5ml) at 4 ˚C and 50 ˚C at pH 6.5. Enzyme catalyzed reactions often exhibit a special form of kinetics, called Michealis-Menten kinetics, which are characterized by hyperbolic relationship between reaction velocity (V) and substrate concentration (S). Normal enzyme kinetic values are measured under steady-state conditions and such conditions usually prevail in the cell. For many enzyme- catalyzed reactions, the kinetics under steady-state can be described by a simple expression known as Michealis-Menten kinetics:

$$V = Vmax\ S / Km+S$$

Where, V is the observed rate of velocity, Vmax is the maximum velocity (at infinite substrate concentration), S is the substrate concentration and Km is the Michaelis-Menten constant which represents the concentration of substrate required to yield half of the maximum velocity of enzyme catalysis. Smaller the value of Km, more strongly the enzyme binds to substrate. The Lineweaver-Burk plot was used to determine the important kinetics parameters of endoglucanase enzyme such as Km and Vmax (Lineweaver and Burk, 1934). The computerized curve was obtained by fitting the values 1/S on x-axis and 1/Vmax on y-axis (Table 1). The y-intercept of such a graph denotes 1/Vmax, while the x-intercept represent -1/Km. The turnover number, the number of substrate molecules converted into products per unit time per molecule of enzyme, was also obtained by dividing the values of Vmax by the amount of enzyme used in the experiment (Table 2). Typical turnover number values ranges from $10^2$ to $10^3$ $S.^{-1}$

**Table 1**

Cellulase activity (µmol /mg protein) of *T. terrestre* at 4°C and 50°C from various CMC concentrations

| S No. | CMC (mg/0.5ml) | 1/S | Cellulase activity (µmol /mg protein) | | 1/V | |
|---|---|---|---|---|---|---|
| | | | 4˚C | 50˚C | 4˚C | 50˚C |
| 1 | 0.16 | 6.25 | 25 | 32.00 | 0.04 | 0.0312 |
| 2 | 0.33 | 3.00 | 31.5 | 37.50 | 0.0317 | 0.0266 |
| 3 | 0.50 | 2.00 | 46.25 | 53.12 | 0.0216 | 0.0188 |
| 4 | 0.66 | 1.50 | 50 | 66.25 | 0.0210 | 0.0150 |
| 5 | 0.83 | 1.20 | 52.50 | 69.37 | 0.019 | 0.0144 |

**Table 2**

Turnover Number of enzyme cellulase at 4˚C and 50˚C temperature

| S No. | CMC (mg/0.5ml) | Turnover Number (mol/0.5ml enzyme/30min) | |
|---|---|---|---|
| | | 4˚C | 50˚C |
| 1 | 0.16 | 50 | 64 |
| 2 | 0.33 | 63 | 75 |
| 3 | 0.50 | 92.5 | 106.24 |
| 4 | 0.66 | 100 | 135.50 |
| 5 | 0.83 | 105 | 138.74 |

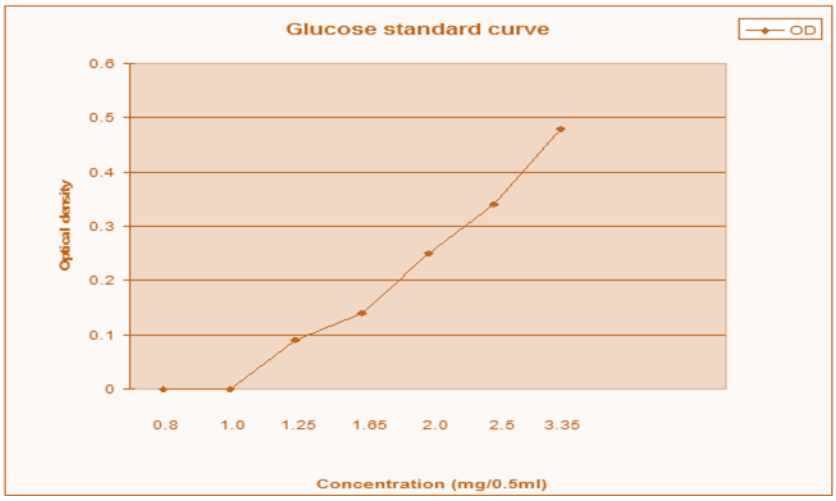


**Figure 1**
Glucose standard curve

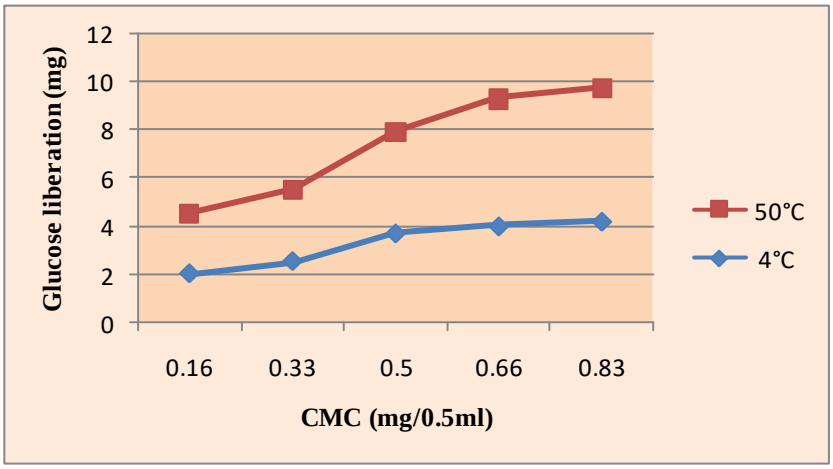


**Figure 2**
Glucose liberation (mg) at 4°C and 50°C with increasing concentration of CMC (mg/0.5ml)

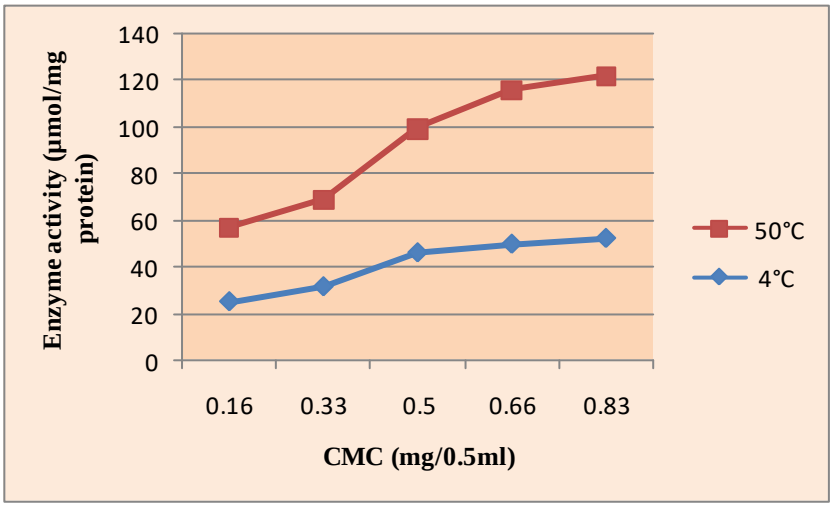


**Figure 3**
Enzyme activity (µmol/mg protein) at 4°C and 50°C with increasing concentration of CMC (mg/0.5ml)

## 3. RESULTS AND DISCUSSION

Among an array of fungal strains isolated during rainy, spring and winter seasons from the rhizosphere of *Juglans regia* L., geophilic fungus *Trichophyton terrestre* was isolated only during the winter season and screened for the production and activity of the enzyme endoglucanase. The enzyme secreted by the fungal species in culture solution at 32˚C was purified to homogeneity. All enzymes that act upon the cellulose are deemed to move about the surface in a random walk (Nimlos et al., 2007; Ting et al., 2009) in steps equating to the dimensions of one glucose molecule (5 A) per time-step. The activity of the enzyme was assayed in the carboxymethyl cellulose solution with the increasing substrate concentrations. Glucose production by enzyme participation was determined at 4 ˚C and 50 ˚C by DNS method thereby generating a reddish brown colour for amino compounds (Vancov and Keen, 2009). The glucose liberation in enzymatic reaction with the chromogenic agents occur in the reaction. The absorbance was measured by the spectrophotometeric method at the wavelength of around 540 nm (Figure 2) (Coleman et al., 2007). Enzyme activity with 0.83mg/0.5ml CMC concentration was found to be 52.5 |mol /mg protein and 69.37 |mol /mg protein at 4 ˚C and 50 ˚C respectively (Figure 3). The enzyme remained active at a wide range of temperatures. A broad temperature optimum was observed for the enzyme endoglucanase ranging from 4 ˚C to 50 ˚C. However, the enzyme has been found to be active at 100 ˚C (Nataraja et al., 2010). The turnover number was observed at varied CMC concentrations. The maximum turnover number at 4 ˚C and 50 ˚C was found to be 105mol/0.5ml enzyme/30minutes and 138.74 mol/0.5ml enzyme/30minutes respectively (Figure 4), when the concentration of CMC was 0.83 mg/0.5ml.

The Km value of enzyme endoglucanase was determined at 4 ˚C and 50 ˚C which was found to be 0.312mM and 0.67mM respectively (Figure 5, 6). Being the fingerprint of an enzyme, Km value is considered the most important criterion to evaluate the enzyme for various uses. Km value indicates the higher efficiency of the enzyme. Lesser the Km value, higher the efficiency of an enzyme. The enzyme was found to be more active at 4˚C temperature, when the concentration of CMC was 0.83mg/0.5ml. Intercept or 1/Vmax at 4 ˚C and 50 ˚C was found to be 0.014

min/mM and 0.010min/mM respectively. The slope (Km/Vmax) was found to be 0.0043mM/min and 0.0067 mM/min at 4 ˚C and 50 ˚C respectively. The maximum activity (Vmax) determined at 4 ˚C and 50 ˚C was 71.42 |mol/mg protein and 100 mol/mg protein respectively. The determination of the kinetic parameters for CMC hydrolysis reveals a fascinating phenomenon of kinetic optimization at different temperatures. The activity of the enzyme at low and high temperature can be viewed as the main physiological adaptation to cold and high temperatures at the enzyme level, as it compensates for the reduction of chemical reaction rates induced by low and high temperatures (Feller and Gerday 2003; Carrard et al., 2000). Because of the extreme molecular stability of endoglucanase at wide array of temperatures, *Trichophyton terrestre* forms an interesting enzyme source for industrial applications such as in textile industries (Gusakov et al., 2000; Belghith et al., 2001), detergents industries (Maurer, 1997), pulp and paper industry (Buchert et al., 1996), food industry (Galante et al., 1998), and in improving digestibility of animal feeds (Lewis et al., 1996). The enzyme thus accounts for a significant share of the world enzyme market. In addition to the above applications, endoglucanses are also employed in generation of antibacterial chito-oligosaccharides which may well be used in food preservation (Liu and Zhu, 2000), immuno-modulation (Tsai et al., 2000) and as potent antitumor agents (Wu and Tsai, 2004). In the biocatalyzed-reaction networks, inhibitory effects of the reaction intermediates play an important role in determining the enzymatic kinetics. The intrinsic reaction kinetics of enzymatic cellulose hydrolysis is also subjected to mediation by a host of factors like inhibitory effects of reaction intermediates and enzyme adsorption etc. The soluble products such as cellobiose and glucose have been reported to be the inhibitors of the cellulase complex (Howell and Stuck, 1975; Katz and Reese, 1968; Ghose, 1977; Nisizawa, 1973), and individual enzyme endoglucanase components (Halliwell and Griffin, 1973; Ladisch et al., 1980), cellobiohydrolase (Hsu et al., 1980) and p - glucosidase (Wood and Mc Care, 1975; Gong et al., 1997). Furthermore, p-glucosidase is also inhibited by its substrate cellobiose (Emert et al., 1974; Berghem and Pettersson, 1974). In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan et al., 1999; Abu et al., 2005). The activities of endoglucanses from *Trichophyton terrestre* were greatly influenced by the concentration of the substrate. During the initial stage of hydrolysis cellulose released more amount of glucose to the solution (Andrew et al., 2011). It is obvious that the concerned mechanisms are extremely multifaceted, since the enzyme effect coupled with intrinsic properties of substrate such as degree of polymerization, crystallinity, or accessible surface area which play crest roles in the enzymatic degradation. The optimal pH of 6.5 for the endoglucanse activity is in conformity to that of Catriona *et al.*, who reported a broad pH range of 5.0 to 7.0 over which the cellulases were highly active. Bok *et al.*, (1998) also reported a pH range of 6.0 to 6.6 for two thermostable endocellulases from *Thermotoga neapolitana*. The physicochemical or ecological conditions existing during the enzyme action need to be taken into account to gain a better understanding of any experimentation. Specifically, the substrate composition, crystallinity, and recalcitrance (Fierobe et al., 2002; Himmel et al., 2007; Jeoh et al., 2006) have a distinctive influence on the mechanism and dynamics of cellulose strap and hydrolysis (Woodward et al., 1992; Be'guin and Aubert, 1994). Cellulases especially endoglucanses isolated from different sources have a wide range of properties depending on their sources. Industrial enzymes working at different temperatures allow technologists to develop processes which closely approach the gentle and efficient processes in nature. The biocatalysts which remain active at cold conditions find their place in chemical synthesis and transformation, bioremediation of contaminants and clean-energy production, confirming and reinforcing the potential of this technology for environmental purposes. The maximum activity (Vmax) was determined at wide range of temperatures. The most important finding was the extraordinary level of tolerance at low and higher temperatures along with a great turn over number. The enzymes showing activity at wide range of temperatures are today the enzymes of choice for microbiologist, biochemists, biotechnologists and industrialists (Kazem, 2012). Endoglucanases active at low and high temperatures are promising enzymes to reinstate the conventional enzyme processes. There is an immediate need for the selection of ideal endoglucanses to retain the activity at extreme range of temperatures which may be utilized in a variety of fields in order to obtain the best results. In this direction, *Trichophyton terrestre* could be used as a prospective source of the enzyme. Extensive research is vital to unknot the full potential of such micro-organisms.

## 4. CONCLUSION

The present study, confirmed that the endoglucanases isolated from *Trichopyton terrestre* can tolerate a wide range of temperatures 4˚C and 50˚C which enables this fungal species to survive in the extreme environmental conditions of northern India, where the winter temperature goes down to -20˚C and touches the upper limit of 37˚C in the summer season. Such a great fluctuation in the temperature occurs only in few regions of India which might be the reasonable factor for the restricted distribution of *Trichophyton terrestre* in Indian soils. The biocatalysts which remain active at cold and hot conditions are desirable in industrial processes. However, an extensive research work is required to overcome several bottlenecks such as less explored biodiversity of psychrophilic

and thermophilic microbes, low activity and stability in terms of turnover number of enzymes under varied environmental conditions.

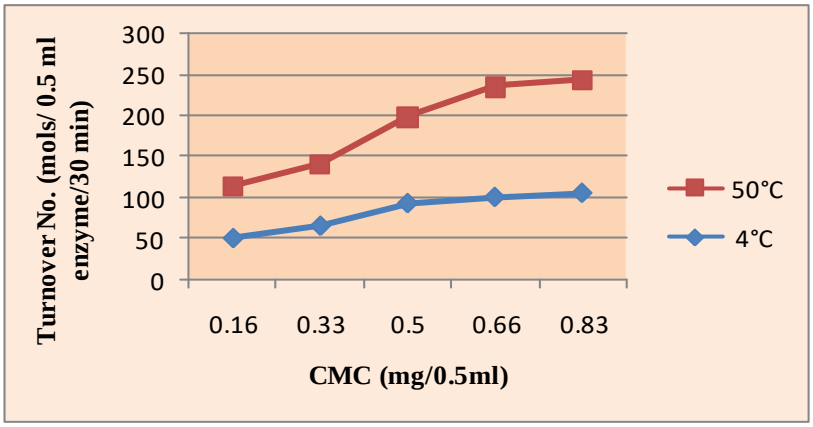


**Figure 4**

Turnover number (mols/ 0.5 ml enzyme/30 min) at 4°C and 50°C with increasing concentration of CMC (mg/0.5ml)

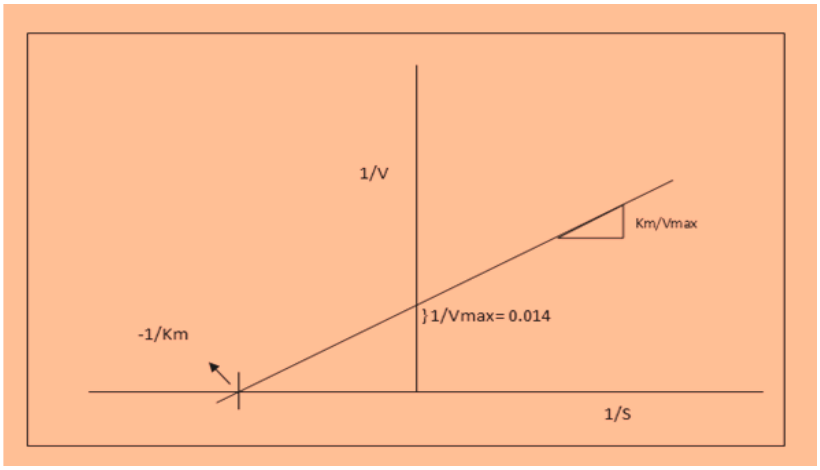


**Figure 5**

Km determination at 4°C

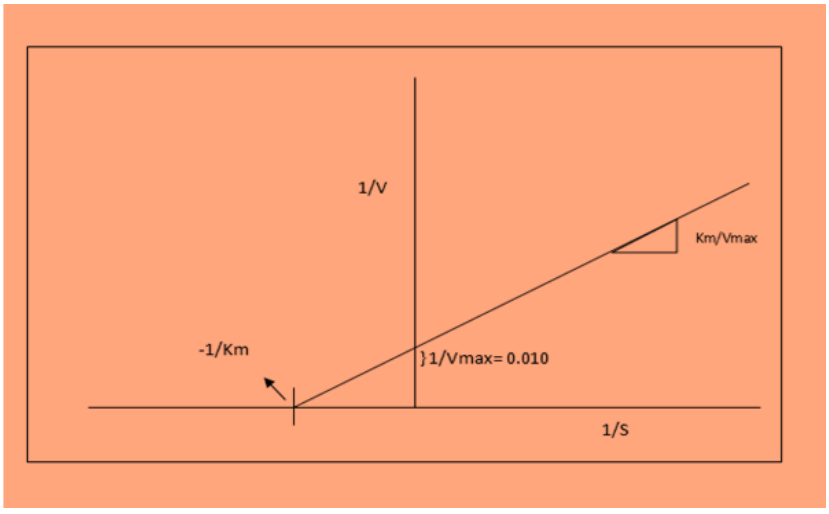


**Figure 6**

Km determination at 50°C

**ACKNOWLEDGEMENT**

The authors are gratified to the Principal, Govt. Science and Commerce College Benazeer, Bhopal- 462 008 (MP), India for providing all the requisite infrastructural support and laboratory facilities with regard to complete these experiments, which were purely funded by authors.

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