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Immobilization of thermostable, bacterial cellulase from *Stenotrophomonas maltophilia* in agar-agarose matrices and its characterization

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Abstract

Cellulase the biocatalyst for conversion of cellulose into simple sugars is gaining global economic importance owing to its wide application in various industrial and clinical domains. Commercial cellulase is preferred to be stable in adverse conditions and to be recovered from the reactor once the process is done. Hence, a search for such a cellulase still exists even though it was discovered six decades ago. In this study, purified thermostable cellulase from *Stenotrophomonas maltophilia* was immobilized on an agar-agarose matrix, and the properties of immobilized cellulase were studied. The optimum temperature and pH for immobilized enzyme activity were found to be 50°C and 8.0 respectively. The immobilized enzyme exhibited its stability at much wider alkaline pH ranges and higher temperatures even after 24 hours incubation. Km and Vmax values of the immobilized enzyme were 6.618 mg/ml and 131.578 µmol/min/mg of protein respectively. Both free and immobilized forms of enzymes were inhibited significantly by Hg^{2+} metal ion and the activity of the latter was affected in the presence of detergents and additives at higher concentration. The agar-agarose immobilized enzyme could be reused up to 5 repeated cycles and it is stable for at least 1 month when stored at 4°C. Hence this immobilized cellulase with good storage stability than the soluble one can be considered for commercial applications.

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Introduction

Lignocellulosic biomass is the most abundant renewable energy source in the natural environment predominantly as agricultural wastes and certain industrial effluents that can efficiently be utilized either as a major source of energy feedstock or as a raw material for the production of high-value chemicals (Kim *et al.*, 2003; Cherry and Fidantsef, 2003). Cellulose, a polymer of β-1,4 linked glucose unit, is a major polysaccharide constituent of plant cell wall (Wen *et al.,* 2005). Biological conversion of cellulose into glucose requires the synergistic action of three enzymes, including endo-β1,4- glucanase, cellobiohydrolase and β-D- glucosidase (Perez *et al.*, 2002). Cellulase has been potentially utilized in leather, textile, agriculture, food, paper and pulp industries yielding high commercial value (Bhat, 2000; Kim *et al.*, 2005). The global market value of cellulase is one thousand five hundred million US$ in 2017 and it is estimated to reach two thousand three hundred million US$ in 2025 according to Global Cellulase (CAS 9012-54-8) Market Research Report 2018.

Besides the high cost of enzyme purification and isolation, the main hurdle is its vulnerability to harsh environmental conditions which leads to limited operational lifetime and difficulty in recovery in the active form (Krajewska, 2004; Guisan, 2006; Roger A., 2007). However, these problems can be surmounted by means of immobilization which is Enzyme immobilization is incarceration of an enzyme within an inert or insoluble material, so as to increase resistance to harsh environmental conditions. Cellulase and its individual constituents have already been immobilized on a variety of solid support materials (Xuepu *et al.*, 2005; Khoshnevisan *et al.*, 2011; Lupoi and Smith, 2011; Alahakoon *et al.*, 2012). Immobilization of enzyme by entrapment method can be done by very simple procedure and moreover, the cost of immobilization is very low when compared to other methods. In our research, cellulase was immobilized directly on agar- agarose matrices. Agarose is a neutral linear polysaccharide, More correctly, it is called as agar-agar, produced from various red algae belonging to *Gelidium, Gracilaria, Gigartino* and *Pterocladia*. It is made up of the repeating unit of agarobiose (a disaccharide consisting of 1,3-linked D-galacto-pyranose and 1,4-linked 3,6-anhydro-α-L galactopyranose). Being an excellent matrix, it has a high porosity, which leads to high capacity for protein entrapment. Uncharged methyl groups, low melting temperature and low cost adds enables it to be an immobilizing agent. In our current study, cellulase produced using *Stenotrophomonas maltophilia* using ammonia-treated paddy straw as substrate and purified (Rohini *et al.*, 2017; Tamilanban *et al.*, 2017) was directly entrapped in agar/ agarose matrix, and analyzed for its activity, stability and reusability at different physio-chemical conditions in comparison with the free cellulase.

Materials and methods

Enzyme

Cellulase isolated from *S. maltophilia* and purified using dialysis, ion exchange and column chromatography as mentioned previously (Tamilanban *et al.*, 2017) was used for this current study.

Immobilization of Cellulase On Agarose and Agar gel

Immobilization of cellulase using agarose and agar did as described elsewhere (Prakash and Jaiswal, 2011). In brief, 1% agarose and 4% agar solution were prepared in 50mM phosphate buffer (pH 8.0) by heating at 50°C. After cooling, 1ml of the enzyme was added to 9ml of agarose and agar solution (total volume of the combination being 10ml) and instantly cast on preassembled clean glass plates. Subsequent to solidification, the gel was cut into small pieces (or beads) of 5*5 mm size and washed several times to remove residual free enzyme attached to the gel surface. The gel pieces were stored in 50mM phosphate buffer (pH 8.0) and at 4°C.

Immobilized enzyme assay

Cellulase activity was assayed by the determination of reducing sugar (glucose) released from carboxymethyl cellulose (CMC) as a substrate

(Teather and Wood, 1982). For immobilized cellulase, the reaction mixture contains agarose/agar bead (~3.4mg/bead), 0.5ml of 1% (W/V) of CMC in 50mM sodium citrate buffer (pH-4.8) was incubated at 50°C for 30mins. The resulted reducing sugar was determined by DNS method (Miller, 1959) using D-glucose as standard. Enzyme activity was determined in terms of the international unit (IU) which is defined as the amount of enzyme required to release 1μmol glucose per ml enzyme solution.

Protein estimation
Protein was estimated by Bradford's method (Bradford, 1976) with Bovine Serum Albumin (BSA) as a standard. At room temperature, the solution was incubated for 5mins and the absorbance was measured at 595nm.

Effect of different pH on enzyme activity and stability
The stability of the enzyme was investigated after pre-incubating the enzyme in different pH buffers at room temperature for 24 hours. pH of the test medium was established using 0.05M Citrate buffer (pH 3.0-6.0), 0.05M Sodium Phosphate buffer (pH 6.5-8.0) and 0.05M Tris HCl buffer (pH 8.5-9.0). The enzyme activity was assayed under standard assay conditions. The enzyme activity was recorded (DNS method) at every 4-hour intervals during the 24 hours of incubation.

Effect of different temperatures and temperature stability
The optimum temperature for efficient activity and stability of free and immobilized cellulase enzyme was determined by performing the enzyme activity assay after incubating the enzyme with the substrate (1% CMC) at 20-100°C for 24 hours with 1% CMC in 50mM phosphate buffer (pH-8) under standard assay conditions. The enzyme activity was recorded at every 4 hour interval.

Kinetics of immobilized enzyme
The kinetics of the immobilized enzyme were evaluated using different concentrations of CMC (1-

10mg/ml) in 50mM phosphate buffer (pH 8) at 50°C for 30mins. The Vmax and Km values were determined from Line weaver - Burk plot.

Effect of metal ions
The effect of metal ions such as Ca^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Zn^{2+} and Co^2 on the immobilized cellulase was studied at a 50mM concentration. Enzyme assay was carried out for 1 hour at room temperature in 50mM phosphate buffer (pH-8). Metal ions along with CMC and without enzyme were kept as control and the relative activities were determined.

Effect of detergents and additives
The effects of detergents on immobilized cellulase were studied at two different concentrations. Detergents such as SDS, Triton X-100 and Tween-20 were used at concentrations of 0.1% and 1% and it was pre-incubated at 50°C with enzyme for 30 mins. Similarly, in order to study the effect of other additives on the enzyme activity, β-mercaptoethanol, EDTA and urea were used at concentrations of 1mM and 10mM. The enzyme assay was carried out under standard assay conditions as mentioned earlier.

Storage stability of immobilized enzyme
The effect of long-term storage stability of the immobilized cellulase enzyme was studied by incubating the immobilized at 4°C for a period of 1 month in 50mM phosphate buffer (pH-8). Aliquots were withdrawn at a particular period (5days interval) and the enzyme activities were assayed using the method described previously.

Reusability of the Agarose and Agar immobilized cellulase
Reusability of the agarose- agar immobilized enzyme was studied by assaying the activity using beads several times for CMC (Carboxy Methyl cellulose) hydrolysis by DNS method (Miller, 1959). First sets of activities were considered as 100%, and then the beads were removed and reassayed. After each assay for the enzyme activity, the beads were removed and

washed thoroughly with distilled water prior to the next set of the assay.

Results and discussion

Cellulase immobilization on agar and agarose

Only a very few reports are available for cellulase immobilization on the agar-agarose matrix by entrapment method. This entrapment method protects the enzyme from direct contact with the environment, as a result, it will minimize the effect of gas bubbles, mechanical shear and hydrophobic solvents (Lalonde and Margolin, 2008). Enzyme immobilization by entrapment method improves their utilization if the optimized immobilization conditions were followed (Zhongliang *et al.*, 2012). Hence, in this study, optimized entrapment procedure was followed as mentioned elsewhere (Prakash and Jaiswal, 2011).

Table 1. Effect of metal ions on the immobilized and soluble cellulase.

Metal ions (5mM)	Relative activity of Soluble cellulase (%)	Relative activity of Immobilized cellulase (%)
None	100	100
Hg^{2+}	42 ± 0.13	38.6±1.05
Ca^{2+}	80 ± 0.99	62.4±0.58
Co^{2+}	103 ± 1.15	96.54±0.67
Cu^{2+}	86±0.70	104.15±0.94
Fe^{2+}	94 ± 1.14	69.24±0.45
Mg^{2+}	91 ± 0.54	58.73±0.28
Mn^{2+}	70 ± 1.16	116.24±0.64
Na^+	97±2.27	74.4±1.46
K^+	110±1.5	88.6±0.87
Zn^{2+}	100.5±0.47	92.5±0.68

Effect of pH on immobilized enzyme activity and stability

The effect of pH on the activity of free and immobilized cellulase is shown in Fig.1. The optimum pH for immobilized and soluble enzyme was found to be 8.0 in phosphate buffer. It is interesting to note that both soluble and immobilized enzymes exhibited the same range of optimum pH. Similar results were reported for a cellulase immobilized on magnetic nanoparticle (Kumar *et al.*, 2018) and urease immobilized on chitosan matrix (Barbara, Maciej, and Wiesawa, 1990) did not show any significant change in its optimum pH. In concordance with our results, purified cellulase from *Trichoderma viride* (Iqbal *et al.*, 2011) and immobilized recombinant esterase by silica coated Ca-alginate also shown the optimum pH of 8.0 (Gulay and Sanli-Mohamed, 2012).

Table 2. Effect of detergents on the immobilized and soluble cellulase.

Detergents	Relative activity of Soluble cellulase (%)	Relative activity of Immobilized cellulase (%)
Control	100	100
Tween 20 (0.1%)	101.28±0.94	98.21±0.54
Tween 20 (1%)	98.34±1.31	61.34±0.25
Triton X-100 (0.1%)	112.24±0.67	104.68±1.02
Triton X-100 (1%)	104.90±1.24	94.24±0.56
SDS (0.1%)	113.35±1.21	105.34±0.64
SDS (1%)	109.94±0.47	100.48±0.84

The pH stability was investigated after pre-incubating the enzyme at pH-8.0 for a period of 24 hours. Residual enzyme activities were measured at every 4 hour interval. Immobilized enzyme retained its 62% of activity even after 24 hours incubation, but in case of the soluble enzyme, it retained only 50% of activity Fig.2. Our observations are in coherence with the previous reports showing the wide range of pH stability as compared to the free enzyme (Abraham *et al.*, 2014; Tao *et al.*, 2016).

**Table 3.** Effect of additives on the immobilized and soluble cellulase.

Chemicals	Relative activity of soluble cellulase (%)	Relative activity of immobilized cellulase (%)
Control	100	100
Urea (1 mM)	84.66±1.64	64.21±1.04
Urea (10 mM)	64.50±1.28	46.32±0.54
EDTA (1 mM)	84.58±0.58	72.32±1.24
EDTA (10 mM)	79.48±1.42	68.54±1.64
β – mercaptoethanol (1mM)	80.54±1.25	62.84±0.86
β – mercaptoethanol (10 mM)	72.26±0.75	54.36±0.74

This may be because of entrapment of the enzymes, which protects it from severe acidic and alkaline conditions.

Effect of Temperature on immobilized enzyme activity and stability
To investigate the effect of temperature on enzyme activity, enzymes were incubated at different temperatures ranging from 20-100°C. Both soluble and immobilized enzymes exhibited a wide range of optimum temperature and were maximally active at 50°C. The effect of temperature on the activity of free and immobilized forms of cellulase is shown in Fig.3. This present study correlates well with Su *et al.* (2012) report in which, maximum activities were shown at 40°C and 50°C for free and immobilized cellulase.
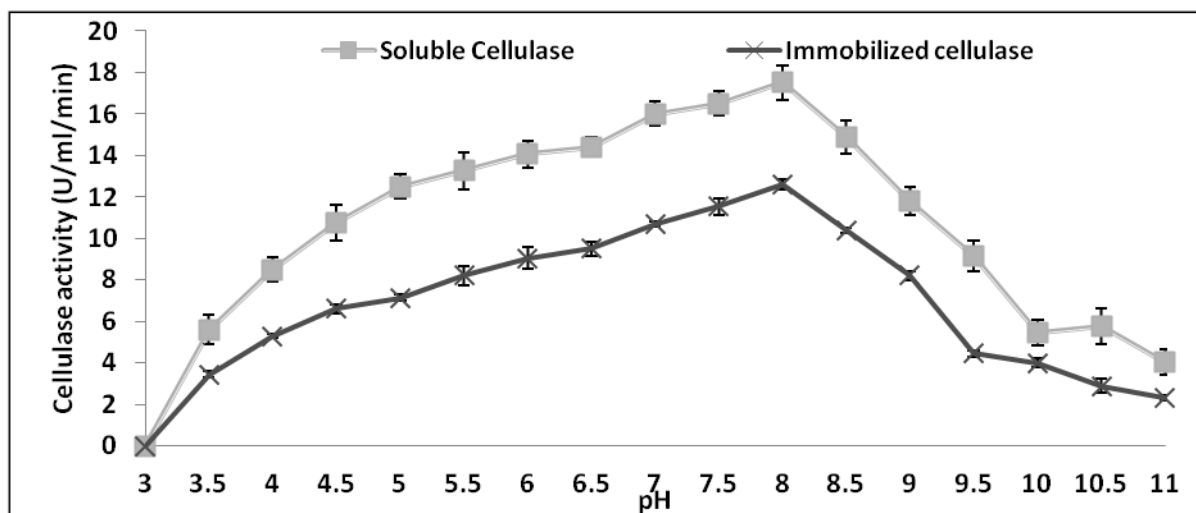
Fig. 1. Effect of different pH values on the immobilized and soluble cellulase.
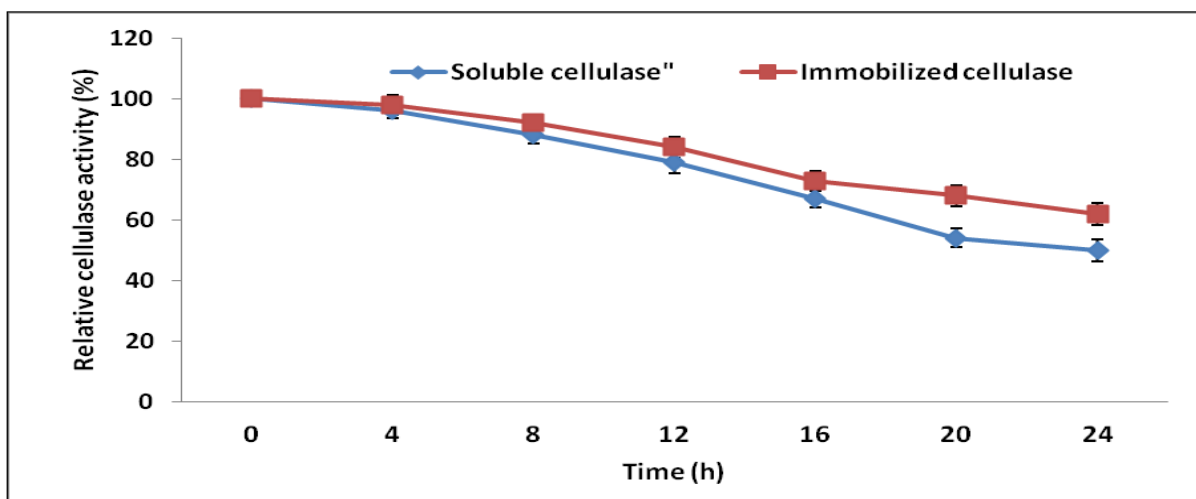
Fig. 2. Stability of the immobilized and soluble cellulase at pH 8.0.

Thermal stability of both forms of enzymes was studied by pre-incubating the enzymes at 50ºC for a period of 24 hours. The immobilized enzyme showed remarkable stability compared with free enzyme. Up to 30% of activity was observed even after 24 hours incubation for immobilized enzyme, but in the case of the free enzyme, only 14% of activity was retained (Fig.4).
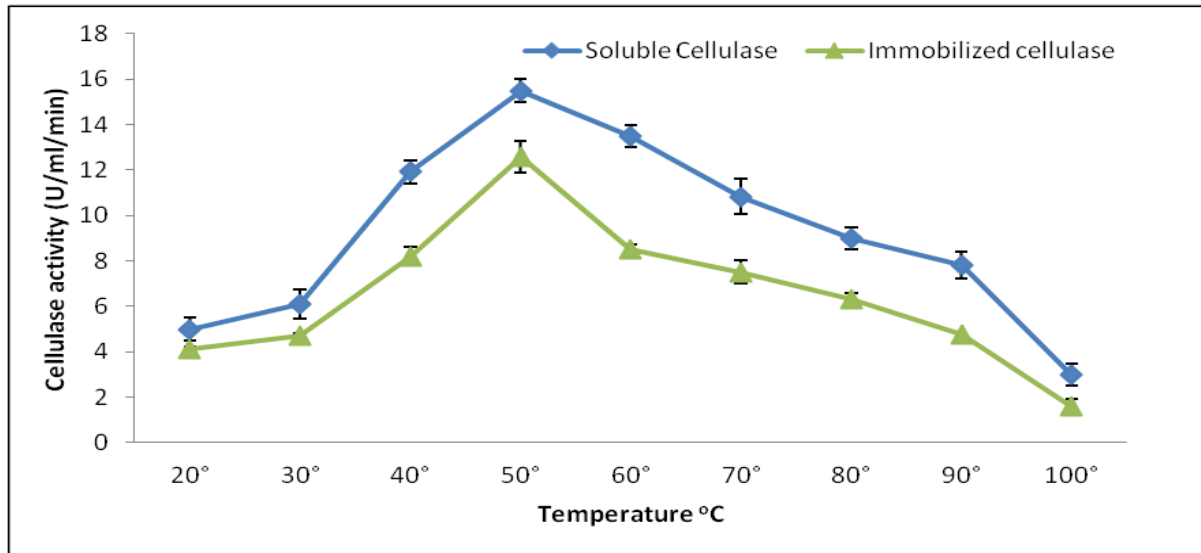

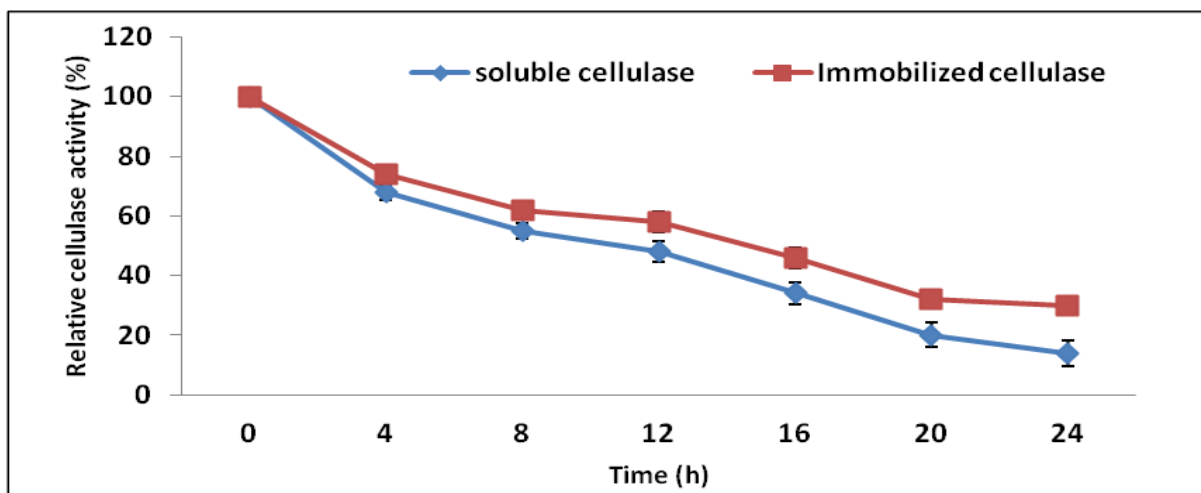**Fig. 3.** Effect of different temperatures on the soluble and immobilized cellulase.

Fig. 4. Stability of the immobilized and soluble cellulase at 50˚C.

Both the enzymes followed same temperature stability up to 8 hours of incubation and after 8 hours, the free enzyme activity started declining sharply, whereas the immobilized enzyme activity was retained without a significant loss for up to 24 hours. Our observations are in line with the previous reports of Jordan *et al.,* 2011 & Huang *et al.,* 2015 showing the increased thermal stability of the immobilized enzymes rather than the free one (Jordan *et al.*,2011; Huang *et al.*, 2015). It may be contributed by the restriction in the conformational movement of the immobilized enzyme (Tümtürk *et al.*, 2007).

Kinetic parameters of the enzymes
The Km and Vmax of soluble and immobilized cellulase were calculated using different concentrations of Carboxy Methyl Cellulose (CMC**).** Enzyme activities were measured under standard assay conditions as discussed earlier and cellulase activity (U/ml/min) against the concentration of CMC (mg/ml) was plotted (Fig. 5).
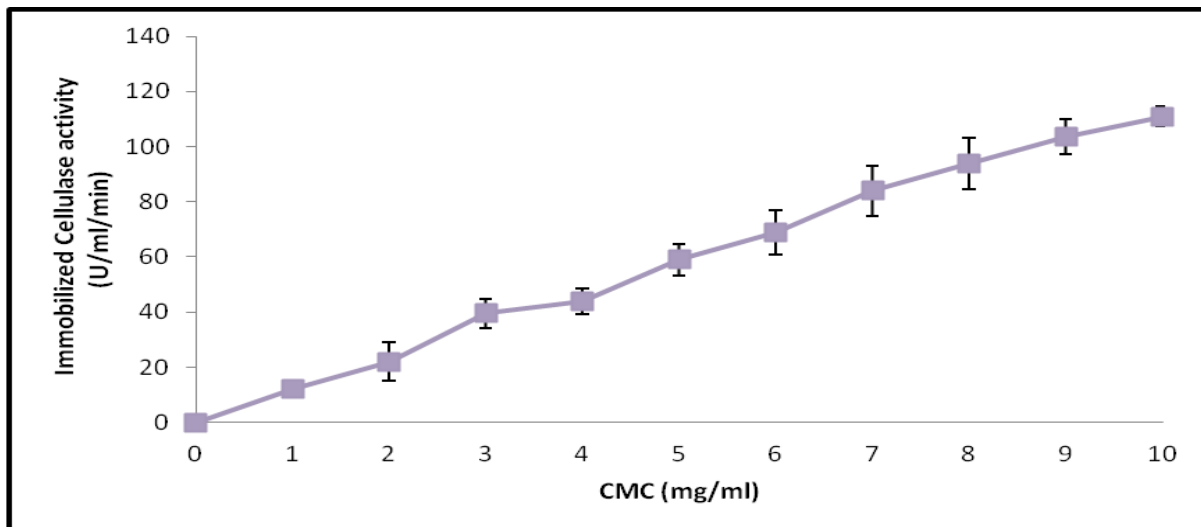
Fig. 5. Effect of substrate concentration (CMC) on the activity of the immobilized cellulase enzyme.

Fig. 6 shows a Line weaver-Burk plot of the immobilized cellulase. The Km and Vmax values from the Line weaver-Burk plot and are found to be 6.61mg/ml and 131.57µmol/min/mg, respectively. Km and Vmax of the purified soluble cellulase were found to be 5.41mg/ml and 161.29 µmol/min/mg (Tamilanban *et al.*, 2017). The increases in the Km and a marked decrease in the Vmax of the immobilized cellulase suggested that some amino acids involved in catalysis are also participating in hydrophobic interaction with the matrix. Similar kind of results was also observed during the immobilization of α-amylase enzyme in Agar-agrose and chitinase enzyme by Acrylamide-Co- Acrylic Acid method (Prakash and Jaiswal, 2011; Prasad and Palanivelu, 2013). Similarly, increased Km value of immobilized enzyme rather than the free one was reported previously (El-hadi *et al.*, 2014).
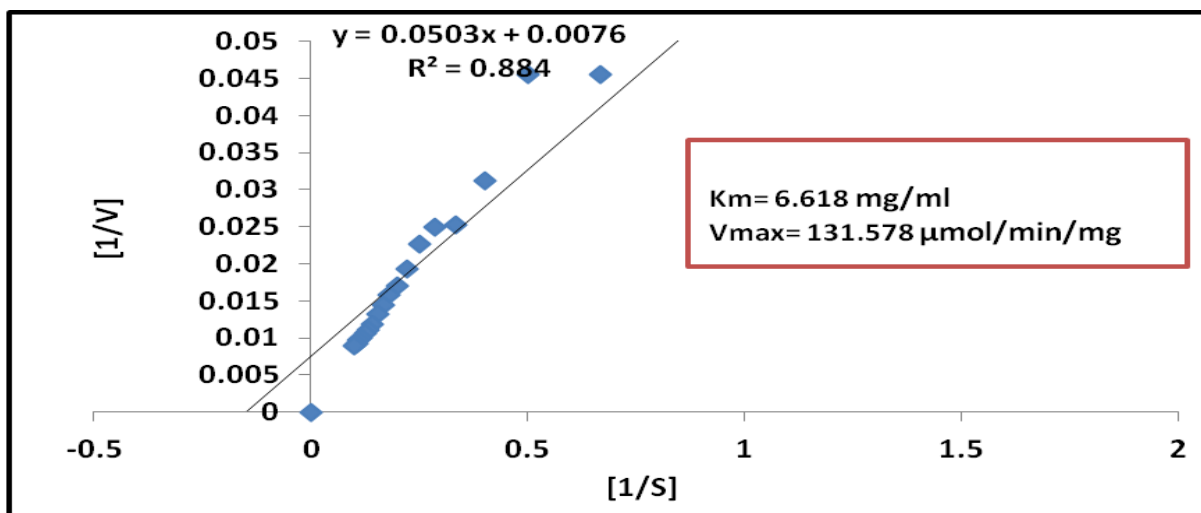

Fig. 6. Double reciprocal plot for determining the Vmax & Km values of the immobilized cellulase enzyme.

Effect of metal ions
As a co-factor, metal ions play a major role in the determination of enzyme activity. Usually, metal ions are known to participate in catalysis reaction, but it is also involved in stabilizing the enzyme-substrate binary complex. The effect of metal ions (5mM) on immobilized cellulase was studied by pre-incubating the enzyme with individual metal ions at 50°C for 30 mins followed by standard assay conditions. Enzyme activity was found to be enhanced by some of the metal ions they are Co^{2+}, Na^+, K^+ and Zn^{2+} for soluble enzyme Mn^{2+} and Cu^{2+} for the immobilized enzyme

(Table 1). Previously also similar enhancement of immobilized enzyme activity by Mn^{2+} was reported in the chitinase enzyme immobilized using Acrylamide-Co- Acrylic Acid (Prasad and Palanivelu, 2013).

Effect of detergents and additives
The effect of detergents on the free and immobilized cellulase was studied. In the presence of surfactants, the free enzyme activity was increased at 0.1% level.

Whereas in the case of an immobilized enzyme, a higher concentration of Tween-20 (1%) decreased the enzyme activity than the soluble enzyme (Table 2).

The outcome of various additives such as Urea (Denaturant), β-mercaptoethanol (Inhibitor) and EDTA (Chelator) was also studied under standard conditions.
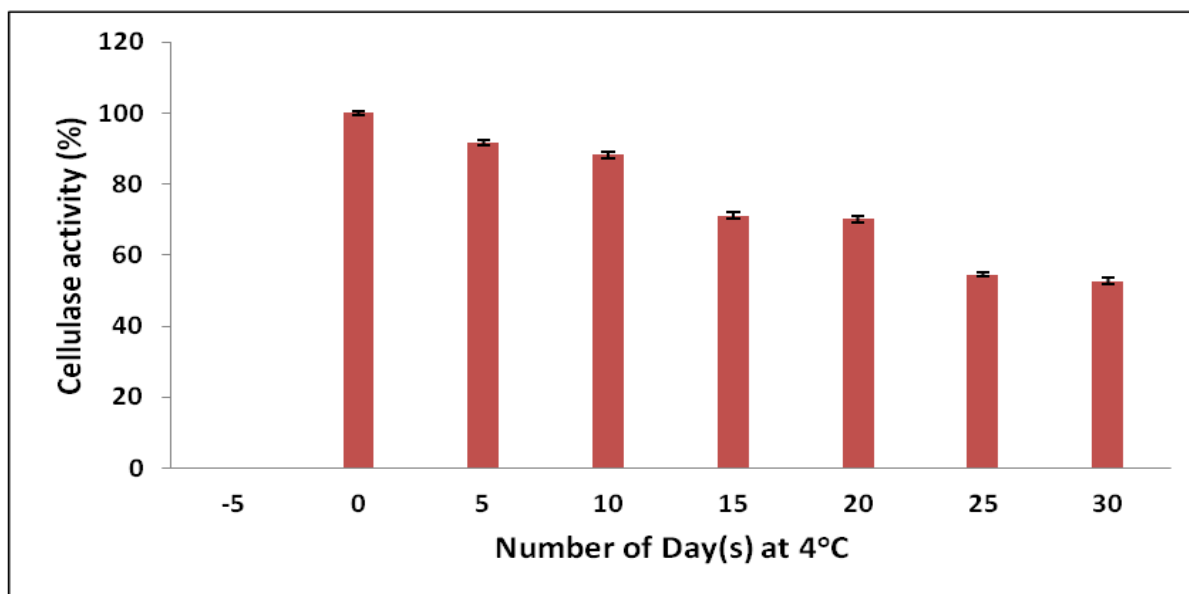

Fig. 7. Storage stability of immobilized cellulase at 4°C in 50mM phosphate buffer (pH-8) for 1 month.

In this study, no significant differences were observed between the immobilized and soluble enzyme activity on the different concentrations (1mM & 10mM) of additives (Table 3).

Long-term storage stability of the immobilized enzyme
The effect of long-term storage on the stability of the immobilized cellulase enzyme was studied by incubating it for a period of one month in 50mM phosphate buffer (pH-8) at 4°C. Aliquots were taken at intervals (5days) and the activity of the immobilized enzyme was assayed under the above mentioned standard assay conditions. Agar-agarose immobilized cellulase lost only about 40% of its original activity over the period of 30 days at 4°C (Fig.7). Previous studies have shown retention of 80% enzyme activity in the immobilized enzymes while the same was 71% for the free enzymes after 6

weeks of incubation (Dinçer and Telefoncu, 2007; Tumturk *et al.*, 2008; El-hadi *et al.*, 2014).

The results clearly indicated that the immobilized enzyme displays good storage stability at refrigeration conditions and hence it can be considered for commercial applicability

Reusability of the immobilized cellulase
The immobilized beads retained its activity up to 5 cycles at ~40% level. This result is in accordance well with a result of Prakash & Jaiswal (Prakash and Jaiswal, 2011). Fig .8 represents the reusability of immobilized cellulase enzyme, which shows a subsequent decrease in enzyme activity after each cycle. This may be because of denaturation and physical loss of enzyme from the carrier (Dey *et al.*, 2002).
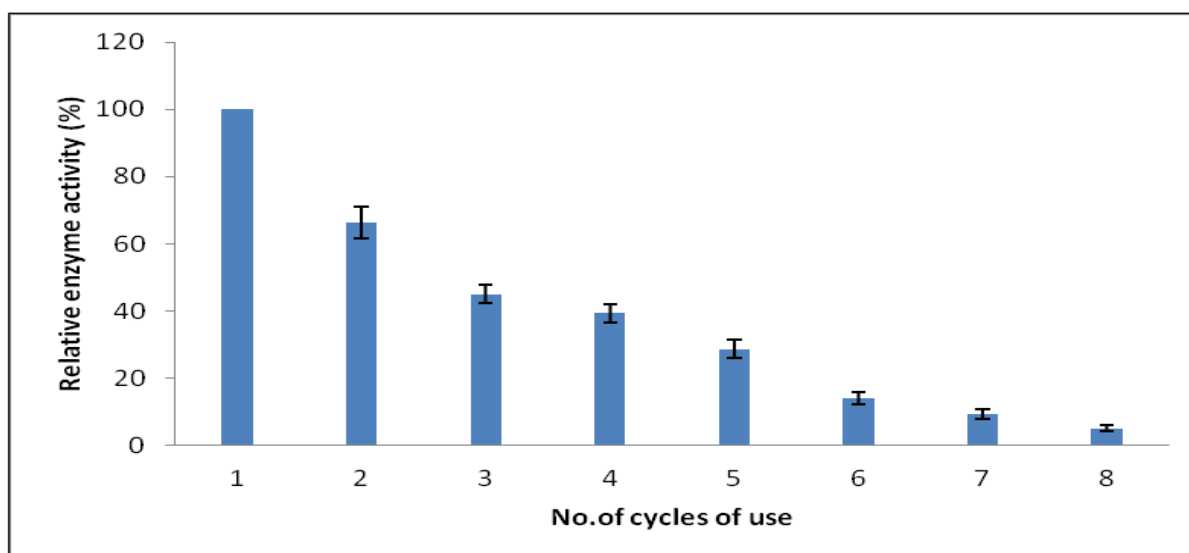
Fig. 8. Reusability of agar-agarose immobilized cellulase.

Conclusion

Both agar and agarose are easily available and inexpensive matrix for immobilization and it is known as a good supporting material for the enzyme. All the properties of immobilized enzyme were characterized, and it was more superior to the soluble enzyme. The optimum pH for the immobilized enzyme was 8, showing its capability for use in detergent industries. The immobilized cellulase enzyme showed higher thermal and storage stability. The reusability of the immobilized enzyme showed its potential in the continuous hydrolysis of CMC (Carboxy Methyl Cellulase) upto 5 cycles in an effective manner.

http://dx.doi.org/

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