# Industrially relevant cellulase production by indigenous thermophilic *Bacillus licheniformis* TLW-3 strain: Isolation-molecular identification and enzyme yield optimization

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**Abstract**: Cellulases are the third largest single industrial bio-robots. These enzymes are employed in industries like pharmaceutical, textile, food processing, paper recycling and detergent manufacturing. In order to produce broadly diversified cellulases, microbes (both bacteria and fungi) have been exploited. Different ecological niches have already been explored for the isolation of cellulolytic microbes. However, there have been no remarkable reports viz a viz to the hot oven ash (for cellulolytic bacterial flora). In this regard, a *Bacillus* strainTLW-3 was isolated and selected for CMCase production and optimization. The strain was identified as *B. licheniformis* TLW-3 through 16S rDNA sequencing that was submitted to Gen Bank with accession numberKY440432. The isolate growth and CMCase production conditions were optimized to get the maximum CMCase yield. The highest growth and maximum CMCase production by *B. licheniformis* TLW-3 were recorded at pH 7 and 50ºC, after the incubation period of 72 (hour) at 150rpm. Studies on the various nitrogen and carbon sources on CMCase production showed that the medium having 1% peptone, 0.5% yeast extract and 1% CMC can significantly enhance the enzymatic yield as compared to other (studied) sources. EDTA, Tween-20 and Tween-80 acted as inhibitors for the enzyme production. The present study holds the conviction that the (reported) organism could directly be applied to produce industrial thermophilic CMCase.

**Keywords**: Hot oven ash, *Bacillus* TLW-3 strain, thermophilic CMCase, optimization, catabolite repression, DNS assay.

## INTRODUCTION

At present, the world is using non-renewable energy resources at a rate higher than their productio. Non-renewable sources take millions of years to be used as energy sources; they are in depletion mode, hence showing the due concern regarding the obvious energy crisis. Due to these issues, there has been shift towards the using of better energy substitute (biofuel) that can meet the high demand of energy resources (Sadhu and Maiti, 2013). For the production of biofuels, lignocellulosic biomass sources offer great potential. Several reasons support the use of these raw materials i.e. environment is rich in lignocellulosic biomasses (these sources are cheaper, largely unexploited and environmental friendly as well). The examples include: stems, stalks and leaves of sugarcane bagasse, rice hulls, corn fiber, corn stover, and forest residues of woody crops, industrial and agricultural lignocellulosic wastes such as sawdust, paper pulp, citrus peel waste, paper mill sludge and municipal solid waste (Mosier *et al*., 2005). Besides these sources, Miscanthus, Bermuda grass, Elephant grass, perennial grasses such as Switchgrass are also utilized as crude material for biofuel generation (Maki *et al*., 2009).

Many microorganisms (bacteria and fungi) in nature produce cellulosomes (by anaerobic bacteria) and cellulase (either extra cellular or cell bound form) during the carbon cycle and convert these (photosynthesis) products in to glucose which is ultimately converted in to $CO_2$ (Berner, 2003). Cellulose degradation requires the complex enzyme consortia of glycosidic hydrolases consisting of three cellulase enzymes i.e. exoglucanases, endoglucanases and β-glucosidases (Bhatt *et al*., 2000). Because of obviously diversified applications, cellulases tend to attract the researchers and industrialists alike. Cellulases are applied in textile, food, detergents, leather, pulp, pharmaceutical, agricultural, brewery, waste control and biofuel industries (Shanmugapriya *et al*., 2012). In pharmaceutical industry, cellulases have been used as a digestive supplement to resolve digestive problems inclusive of malabsorption. These enzymes appear to be vital for the healthy cells. Most of these industries prefer the cellulases that are stable at high temperature. Production of enzyme in bacteria is closely regulated and depends on the complex relationship among the different factors like temperature, pH, time course, carbon and nitrogen sources in media and medium composition etc. (Azadian *et al.,* 2017).

The main objective of this study has been to optimize the different growth factors required for the maximum

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cellulase production by the indigenously isolated thermophile bacterium.

## MATERIALS AND METHODS

***Bacterial strain***

The bacterial strain was taken from the previously isolated-maintained strains collection (from ash stove samples) (Kiran *et al.,* 2015).

***Molecular identification***

Identification of *Bacillus* TLW-3 (up to species level) had been carried out by the sequencing of 16S rDNA. Thermo-scientific (genome isolation) kit was used for the extraction of DNA by using following universal primers: forward 5'-AGAGTTTGATCITGGCTCAG-3'; I= inosine, reverse 5'-ACGGITACCTTGTTACGACTT-3'. The parameters of program included initial denaturation for 10/min at 95/°C, denaturation for 15 sec at 95/°C, for 45 cycles, annealing for 1 min at 50/°C, extension for 30 sec 72/°C and final extension for 5/min at 72/°C. The amplified product was analyzed using agarose (1%) gel electrophoresis and sequenced. BLAST was run to search the sequence similarity (http://www.ncbi.nlm.nih.gov/blast/). Mega software (version 6.0.1) was used for sequence alignment and phylogenetic tree construction.

***Enzyme production***

For enzyme production 18 (hour) grown (fresh) culture was inoculated in to a flask containing production medium ($MgSO_4$ 0.025%, $K_2HPO_4$ 0.1%, $FeSO_4$ 0.025%, $MnCl_2$ 0.05%, yeast extract 0.5%, peptone 1% and CMC 1%) and incubated at 50°C and 150 rpm for 48 (hour). The broth was centrifuged and the supernatant was collected and used as the crude enzyme. Enzyme assay was performed by DNS (dinitrosalicylic acid) method. One unit of enzyme is characterized as the measure of enzyme required to discharge 1µmol of the reducing sugar under standard assay conditions.

***Growth curve***

*B. licheniformis* TLW-3 culture (24 hour grown) was inoculated into the 100ml production medium and incubation was done at 50°C and 150 rpm. After regular intervals of 1 hour, the samples were drawn. Isolate growth was measured at $OD_{600}$. CMCase activity assay was also carried out to identify that at which phase the enzyme production was started (Seo *et al.,* 2013).

***Optimization of growth production and parameters***

*Effect of incubation time*

To determine the time required for maximum CMCase production, the media were inoculated with the isolate and incubation was done at 50˚C (at 150 rpm). The fermented medium was taken at regular intervals of 24 (hour) [for 0-120 (hour)] and evaluated for growth and CMCase activity.

***Effect of temperature***

For temperature optimization, the bacterial strain was inoculated in production media and incubated at 150 rpm for 72 (hour) with temperature ranges from 30-70˚C, thereafter, the growth and the CMCase activities were determined.

***Effect of pH***

For the optimum pH determination, the production media pH was adjusted at 3-11 range. The culture was grown (at different pH values) for 72 (hour) at 150 rpm. Enzyme activity and growth were determined after 72 (hour) of incubation.

***Effect of carbon sources***

CMC in the fermentation media was substituted by various carbon sources i.e. glucose, fructose, lactose, maltose, mannitol, starch, raffinose and cellulose. Inoculum was added and incubated for 72 (hour) at 50˚C and the enzyme activity was determined by DNS method.

***Effect of nitrogen sources***

Effect of various nitrogen sources on the CMCase production was studied by replacing the yeast extract and peptone in production media (replaced by 1% beef extract, yeast extract, tryptone, peptone, urea, casein, $NH_4Cl$ and $(NH_4)_2SO_4$). Inoculum was added and incubation was done for 72 (hour) at 50˚C and enzyme activity was measured by DNS method.

***Effect of salts***

Different salts and surfactants ($CaCl_2$, NaCl, $HgCl_2$, T-20, T-80, EDTA and SDS) were added in the production medium at 0.2% concentration to study the impact of salts on CMCase production.

## STATISTICAL ANALYSIS

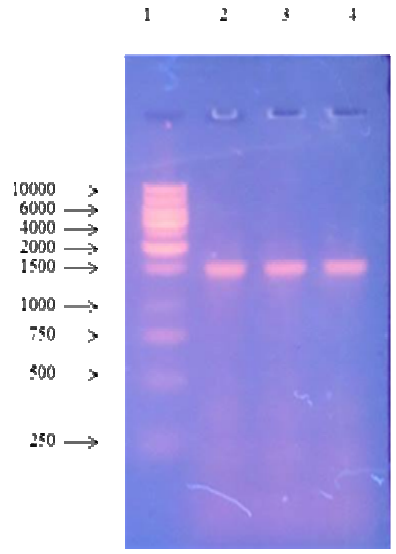
All investigations were run in triplicates and values shown as mean ± standard deviation. Bonferroni test was applied to see whether the values differ significantly or not.

## RESULTS

***Molecular identification of Bacillus sp. TLW-3***

Upon sequencing of the 1.5kb (fig. 1) 16S rDNA (re-amplified) gene composed of 947-nucleotides was obtained from *Bacillus* TLW-3. Then, the sequence (obtained) was matched with the available NCBI sequences by Basic Local Alignment Search Tool (BLAST) and phylogenetic tree (depicted in fig. 2) was constructed using Mega 6 software. This study involved 29 nucleotide sequences. Evolutionary history was inferred by Tamura-Nei model using MLH (Maximum Likelihood) method. Phylogenetic tree shows that the isolate is closely related to the strain *B. licheniformis*

TT102 with high bootstrap value (87%). It confirms that the isolated species is related to the *B. licheniformis* TT102 with 87% bootstrap value. Hence, the studied strain was identified and named as *B. licheniformis* TLW-3. The sequence (16S rDNA) was submitted to NCBI with KY440432 accession number.




Key: Lane 1 shows ladder in bp (Fermentas Life Sciences); Lanes 2 to 4 show the amplified product of 16S rDNA

**Fig. 1**: Agarose gel electrophoresis of 16S rDNA gene of *B. licheniformis* TLW-3

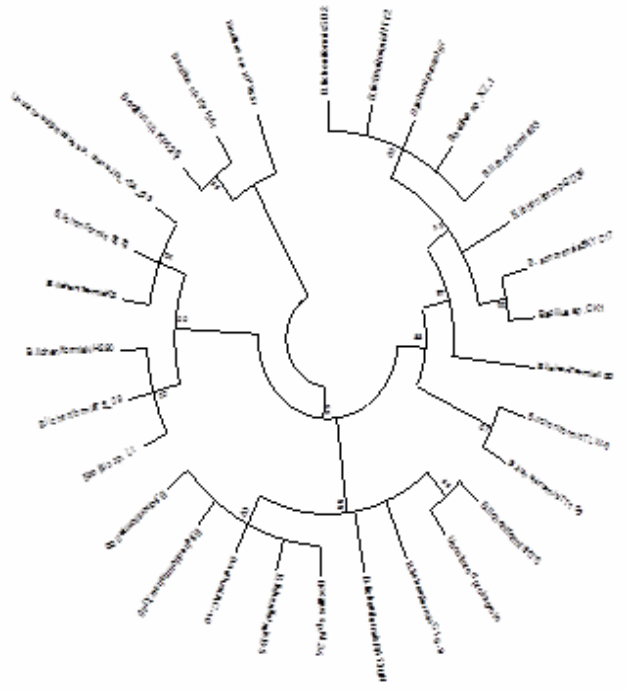


**Fig. 2**: Phylogenetic tree constructed by maximum likelihood approach (MLA) using MEGA 6.0 software.

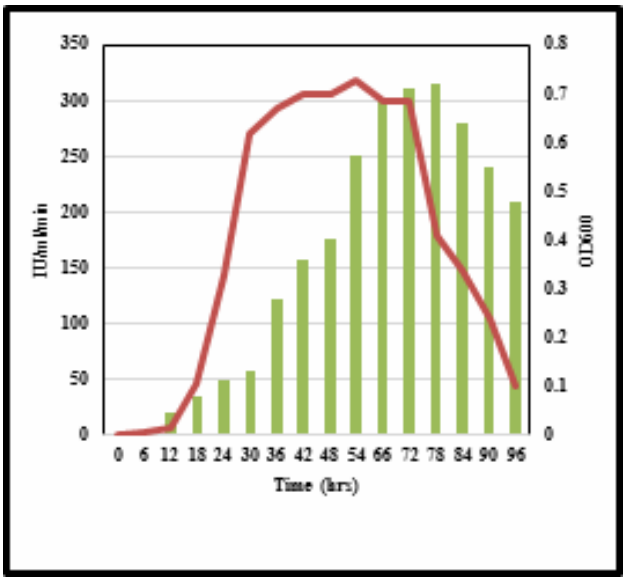
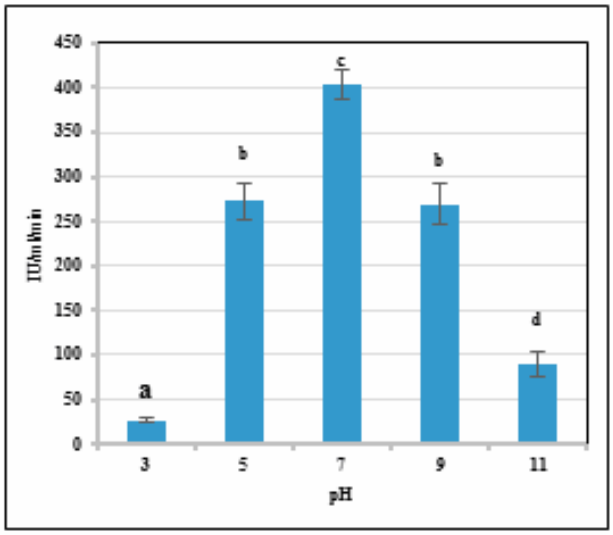



**Fig. 3**: Growth curve of *B. licheniformis* TLW-3 viz a viz to the CMCase synthesis




Key: Same alphabets mean no significant difference was found among the values (Bonferroni test, $p < 0.05$)

**Fig. 4 i**: pH variation effect on CMCase production

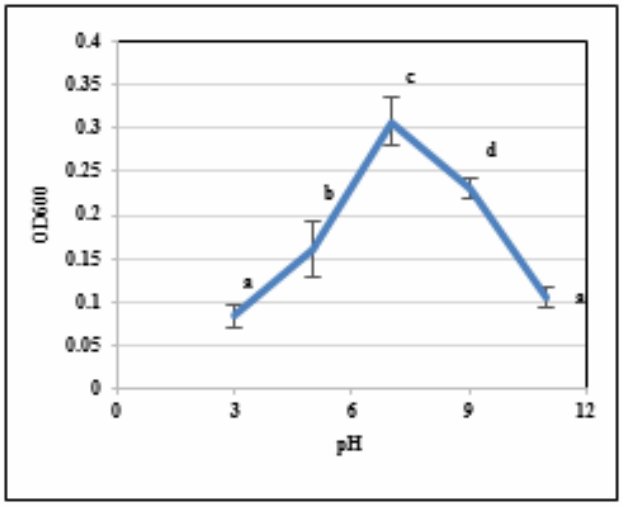
***Growth curve***

The lag phase of *B. licheniformis* TLW-3 (0.8% starting inoculum) terminates after 6 (hour) of incubation, thereafter, the log phase was initiated and ended after 30 (hour) (of incubation). After 72 (hour) (of incubation), the stationary phase was over followed by entering the decline phase. Production of CMCase was initiated in log (growth) phase and increased rapidly concomitant to the cell growth (biomass) increase, which indicated the substrate (CMC) was rapidly hydrolyzed during the log phase. Maximum activity of CMCase was found as the stationary phase ended this activity (CMCase production) by *B. licheniformis* TLW-3 and thereafter was found decreased.

***Optimization of production and growth conditions***

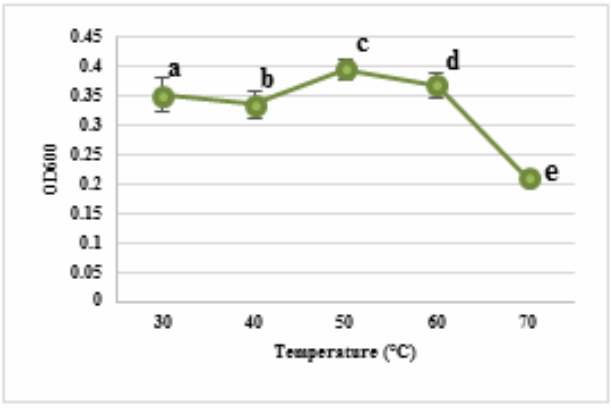
***pH optimization***

Production of CMCase was gradually increased as the pH of the medium increased, showing maximum production at pH 7. However, when the medium pH (initial) turned to alkaline, the enzyme production tended to decrease (fig. 4.i). Same pattern was witnessed for the biomass production (growth) of the studied isolate (fig. 4.ii). Optimum growth (0.3080±0.0267 at $OD_{600}$) and enzyme production (403.89±17.11 IU/ml/min) were found at pH 7 of the medium.




Key: Same alphabets mean no significant difference (found) among the values (Bonferroni test, p <0.05)

**Fig. 4 ii**: Varied pH effect on *B. licheniformis* TLW-3 growth




Key: Same alphabets mean no significant difference (found) among the values (Bonferroni test, p <0.05)

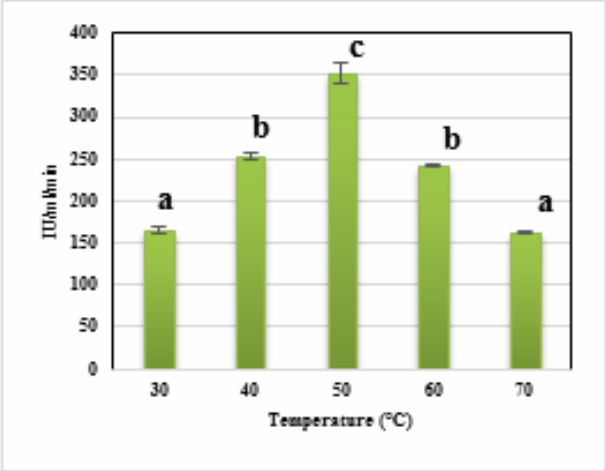
**Fig. 5.i**: Temperature variation and the *B. licheniformis* TLW-3 growth

***Temperature optimization***

The producer culture growth and CMCase production were found maximum at 50°C i.e. 0.39600±0.0173 $OD_{600}$ and 452.47±13.01 IU/ml/min respectively (figs. 5.i and 5.ii). Further increase in temperature led to a gradual decrease in the growth and the CMCase production.

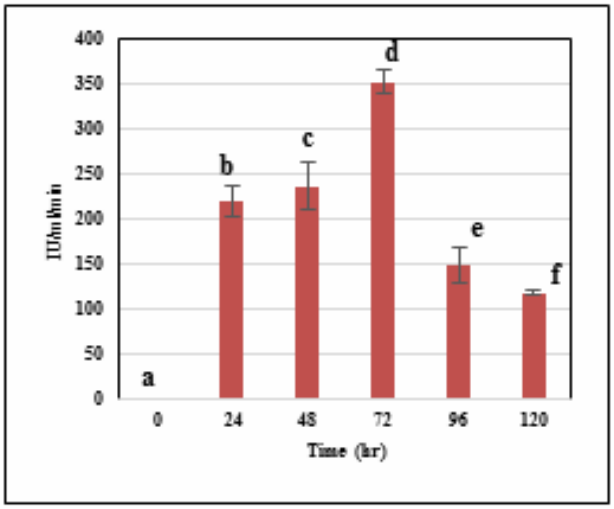
***Incubation time***

At the conclusion of the incubation period 72 (hour), CMCase production and the growth were observed as the maximum i.e. 452.47±13.01 IU/ml/min and 0.327±0.020 $OD_{600}$ respectively (figs 6.i and 6.ii). Thereafter, a gradual decrease in growth rate and the enzyme production was noted.




Key: Same alphabets mean insignificant difference (found) among the values (Bonferroni test, p <0.05)

**Fig. 5 ii**: Temperature effect on CMCase production




Key: Same alphabets mean insignificant difference (found) among the values (Bonferroni test, p <0.05)

**Fig. 6.i**: Incubation (time) effect on CMCase production

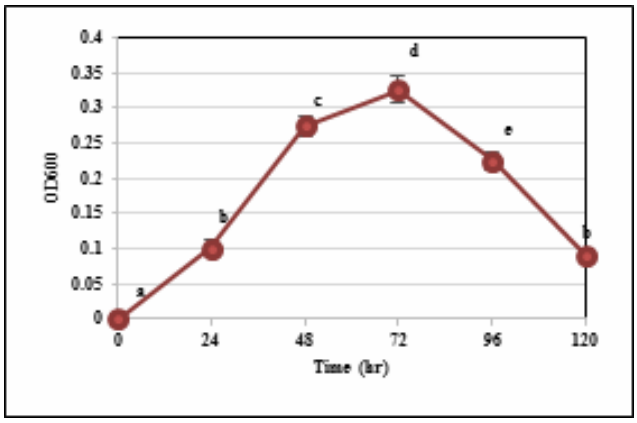
***Optimization of carbon sources***

Accordingly, CMCase production was observed maximum (314.2±18.3 IU/ml/min) in the medium containing 1% CMC as the exclusive saccharide source (fig. 8).

***Nitrogen source optimization***

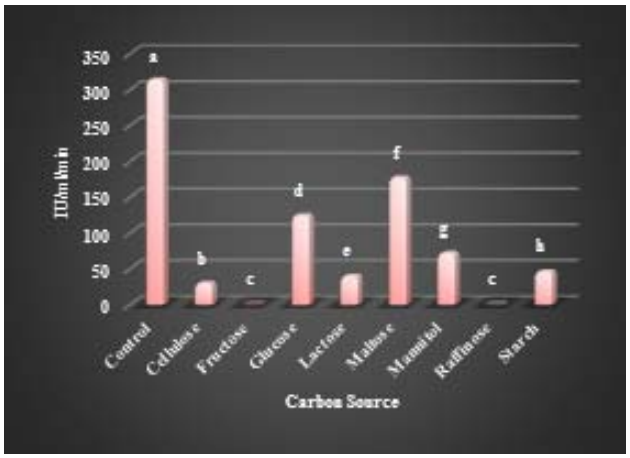
Maximum CMCase production (318.52±11.26 IU/ml/min) was obtained in the control production

medium having 0.5% yeast extract and 1% peptone being the nitrogen sources (fig. 9).




Key: Same alphabets mean no significant difference (found) among the values (Bonferroni test, $p < 0.05$)

**Fig. 6 ii**: Incubation (time) and the *B. licheniformis* TLW-3 growth




Key: Same alphabets mean no significant difference (found) among the values (Bonferroni test, $p < 0.05$)

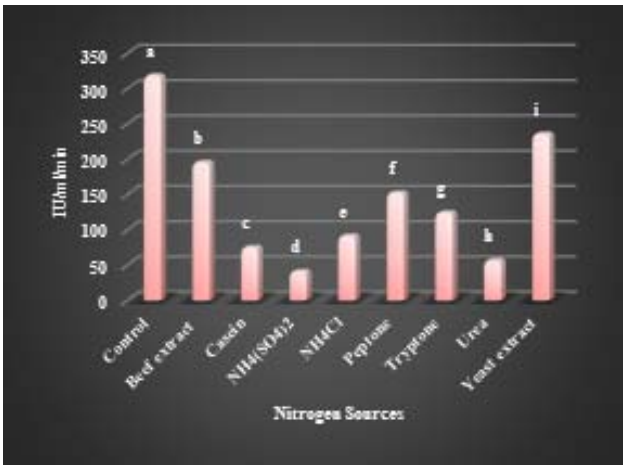
**Fig. 8**: Carbon source effect on CMCase production

***Effect of salts and surfactants***

Maximum enzyme production was observed in the control fermentation medium (not having any salt or surfactant). Accordingly, CMCase production was seen when the medium was amended with 0.2% $CaCl_2$ and NaCl (fig. 10). However, other (tested) salts and surfactants did inhibit the CMCase production, whereas, in the presence of EDTA in the medium, the enzyme production was completely suppressed.

## DISCUSSION

The identification of cellulolytic (indigenous) bacterial isolate was performed with the help of 16S rDNA sequencing as *B. licheniformis* TLW-3. The *B. licheniformis* TLW-3 growth curve indicates that a 0-6 (hour) period was the adjustment period in which the cells prepare themselves for multiplication. After 6 (hour), log phase ensues and lasts up to 30 (hour) of incubation. However, the log phase of the isolate *B. licheniformis* JK7 was spread over a period of 0-16 (hour) of incubation (Seo *et al.,* 2013). The log (growth) phase of the studied strain was lengthier, which may be due to the presence of different nutrients in the medium. The stationary phase of the isolate (*B. licheniformis* TLW-3) terminated after the incubation period of 72 (hour) and thereafter, the isolate entered the death phase. Production of CMCase was started in initial phase of acceleration and enzyme production enhanced with the increment of the cell biomass, thereby indicating that the substrate (CMC) was hydrolyzed actively by isolate during growth phase. A number of researchers support the present results (Ariffin *et al.,* 2008; Saratale and Oh, 2011; Kazeem *et al.,* 2017). Highest level of CMCase production was found at the end of stationary phase. According to Rastogi *et al.* (2010), highest production of CMCase by *Bacillus* sp. DUSELR13 was found by end of the stationary phase. Plus, the isolate (*Geobacillus* WSUCF1) synthesized maximum CMCase (unit wise) as the stationary phase was about to switch over (Rastogi *et al.,* 2010). The CMCase activity by *B. licheniformis* TLW-3 was lowered, which could be explained in the light of accumulation of hydrolysis products, thereby, decreasing the enzyme production (Saratale and Oh, 2011).




Key: Same alphabets mean no significant difference (found) among the values (Bonferroni test, $p < 0.05$)

**Fig. 9**: Nitrogen sources vs. CMCase production

Cost effective production of the cellulose is an important indicator which effects the overall hydrolysis reaction (Singh and Kaur, 2012). Extensive improvement has been carried out to get the maximum yield of cellulase by optimization of factors (affecting the production cost) including growth temperature, initial pH of the medium (and its components), incubation time etc. According to our study, pH 7, 50ºC temperature and after 72 (hour) of incubation, the CMCase production was found maximum. Similar conditions were found true for the highest

biomass (growth) of *B. licheniformis* TLW-3. Other study also reported the maximum growth of *Bacillus* sp. at neutral pH (7.0), while the maximum cellulase yield was noted at pH 8 (Das *et al.,* 2010). For maximum growth, pH 7 was also reported by several investigators (Rasul *et al.,* 2015: Goswami *et al.,* 2016; Khatiwada *et al.,* 2016; Hussain *et al.,* 2017). Temperature of 50ºC was found optimum for cellulase production by many bacterial isolates (Chellapandi and Jani, 2008; Sadhu *et al.,* 2012; Sadhu *et al.,* 2013). Contrarily, Yang *et al.* (2014) found the highest CMCase yield after 24 (hour) by *Bacillus subtilis* BY-2. However, the maximum CMCase yield by *Bacillus subtilis* NASCB-5 was obtained after 72 (hour) (Rathnan *et al.,* 2013).


Key: Same alphabets mean no significant difference (found) among the values (Bonferroni test, $p < 0.05$).

**Fig. 10**: Salts and surfactants vs. the CMCase production

Optimization of medium components is an important factor in fermentation industry. Designing of inexpensive medium to get the maximum cellulase yield may help to reduce the overall enzyme production cost. In this study, maximum yield of CMCase was obtained in the presence of 1% CMC (as an exclusive carbon source). Narasimha *et al.* (2006), Niranjane *et al.* (2007), Sadhu *et al.* (2014) and Premalatha *et al.* (2014) reported CMC (to be the best carbon source) for the highest CMCase yield. In other studies agro-industrial wastes such as rice bran, wheat bran, sugarcane bagasse and rice straw helped for the highest CMCase yield (Heck *et al.*, 2002; Poorna and Perma, 2007; Devi and Kumar, 2017).

Production of CMCase by microbes has been correlated with the presence of varied nitrogenous sources (both inorganic and organic) in the fermentation medium. Present research is suggestive of the fact that yeast extract and peptone presence in the medium appreciably do enhance the CMCase production by *B. licheniformis* TLW-3. Ray *et al.,* (2007) noted maximum cellulase production in the medium that was supplemented with organic nitrogen source. Similarly, *B. cereus* MRK1 (Kumar *et al.,* 2012) and *P. barcinonensis* MG7 (Asha *et al.,* 2012) gave highest CMCase yield in the medium incorporated with yeast extract. However, *B. subtilis* KO (Shabeb *et al.,* 2010) and *B. sonorensis* HSC7 (Azadian *et al.,* 2017) gave the maximum CMCase yield in the production medium added with $(NH_4)_2SO_4$ (as the sole nitrogen source). During this study, the production of the enzyme was same (as that of control) in the presence of $Ca^{+2}$ and $Na^{+}$ ions in the production medium. However, $Hg^{+2}$ ions did inhibit the CMCase production and that may be a direct result of the chelation of the metal particles to be corrosive along these lines, hampering the bioavailability of metal particles that influence the metabolic procedure productivity, active enzyme secretion and the capacity of organisms to tie with particular substrate (McDonald *et al.,* 1996).

## CONCLUSIONS

*B. licheniformis* TLW-3 carries hyper CMCase production characteristic. It produces CMCase in broader pH and temperature ranges. Highest enzyme production was noted at 50ºC, pH 7 and after 72 (hour) of incubation at 150 rpm. CMC (1%), yeast extract (0.5%) and peptone (1%) were similarly found to be the best carbon and nitrogen sources for enhanced enzyme production. However, Tween-20, Tween-80 and EDTA were found to inhibit the CMCase (enzyme) production.

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