Cellulase derived from Chryseobacterium indologenes LA4K isolated from Indonesian agar-agar industry solid waste

I Munifah1*, J Basmal1, R Kusumawati2 and H E Irianto2

1 Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Jakarta, Indonesia
2 Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Jalan KS. Tubun Petamburan VI, Slipi, Central Jakarta 10260, Indonesia

*Corresponding author email: ifah_munifah@yahoo.com.au

Abstract. Hydrolysis process can be a challenging task to perform because the required enzymes are difficult to find and microbial culture is relatively expensive. However, the process can be made more economical by utilizing enzymes which can be produced using substrates derived from local materials. One example of such materials is the solid waste generated by industries involving agar-agar which is obtained from the red alga Gracilaria sp. Chryseobacterium indologenes LA4K is an indigenous bacterial variant which is isolated from agar-agar industry solid waste. The cellulolytic enzyme of C. indologenes LA4K was processed using a medium of 2.5% agar-agar industry solid waste which was incubated for six days and showed an activity of 0.3726 U/mL. Reaction incubation temperature exerted some influence on the activity of C. indologenes LA4K: the process produced the optimum activity of 0.3246 U/mL at the temperature of 40°C, while 30 minutes was determined as the optimum incubation period needed by both enzyme and substrate of C. indologenes LA4K to react. The enzyme entirely lost its activity when heated to the temperature of 80°C. The activity of LA4K cellulase increased with an addition of divalent ions (Mg2+, Ca2+, and Zn2+) and monovalent ions (K+ and Na+). An addition of trivalent ion Fe3+ to C. indologenes LA4K cellulase acted as an inhibitor to its activity. Addition of ethanol as an organic solvent to the supernatant crude enzyme caused the enzyme to become denatured, thus reducing the cellulase activity of its C. indologenes LA4K isolate to 85.47%. The presence of anionic detergent SDS in the crude cellulase of C. indologenes LA4K increased its activity to 105%. Addition of nonionic detergents Triton X-100 and Tween 80 to C. indologenes LA4K cellulase increased its activity to 130% and 110.9%, respectively.

1. Introduction
Algal polysaccharide in the form of agar-agar as food ingredients, medicinal substances, pharmaceutical media, and additive substances is often utilized in various types of industries, such as pharmacy, cosmetics, biotechnology, and textile. Such a wide range of usages has increased the demands for algae as industrial raw material, both in Indonesia and abroad. However, the management of waste generated by the algae processing industry has not attracted much attention. Most of the algal industrial waste only ends up as a pile of organic refuse without being subjected to further, more useful processing. One of many forms of algal industrial waste is solid waste. Waste-based products provide
a potential added value, and this shows that algae processing industry, especially the agar-agar industry, can actually generate more derivative products and obtain more profit from the application of those products.

One of the carbohydrate components of waste generated by the agar-agar production process is cellulose. Cellulose is the structural basis of all plants. Cellulose is the main component of the walls of all plant cells which is composed of up to 10,000 units of glucose in the form of hydroglucopyranose units with the formula \([C_6H_{10}O_5]_n\). According to Kumar et al. (2013) [1], 100% of fresh algae material which has been processed into agar-agar can produce a collection of waste with a holocellulose content of 62 to 68%. Solid waste from agar-agar industry has been found to contain cellulose [2,3], and the cellulose content in the waste can reach 15 to 25% [1].

*Gracilaria sp* is a species of red alga which is commonly used as the main ingredient in agar-agar production. According to a study by Kumar et al. (2013) [1], 40% of cellulose was found in agar-agar solid waste. Agar-agar waste has been used for a variety of uses, such as cattle food, a growth medium for edible mushrooms, and material for particle boards. One of the problems in the utilization of agar-agar waste in a large, industrial scale is its relatively high content of celite. Researchers are still investigating the use of indigenous cellulolytic bacteria which are derived from their original substrate for decomposing agar-agar industry solid waste. Several potential candidates for cellulolytic isolates with a high content of celite have been identified in agar-agar waste [4]. Those isolates are able to better degrade the cellulose of agar-agar waste and to yield derivative products in the form of cellulolytic enzyme and hydrolysis products in the form of simple sugar. Considering these high potentials, the problem of excess solid waste in agar-agar industry can actually be overcome in a more efficient way. The aim of this paper is to identify and describe the characteristics of an enzyme which is produced by one of the cellulolytic bacteria using one type of substrate, i.e. agar-agar industry solid waste.

2. Materials and methods

This research was carried out in the Research and Development Center for Marine and Fisheries Product Processing and Biotechnology (BBRP2BKP) in Jakarta. The source of the substrates used in this study was agar-agar industry solid waste obtained from the red alga *Gracilaria sp*. The solid waste sample was obtained from CV Agar Sari Jaya, an agar-agar factory in Malang, East Java, Indonesia.

For the purpose of this research, the agar-agar industry solid waste took the form of solid residue as the result of the filtering process. The preparation for obtaining agar-agar industry solid waste as substrate involved removing contaminants, grinding the material, and sifting the result until it reached the size of 35 mesh and was free from most of its celite mineral content. The size was gradually reduced until reaching 200 mesh. The ultra-fine agar-agar industry solid waste was then separated from its agar-agar component by means of heating, cooling, and drying through the freeze-drying process, resulting in agar-agar solid waste fiber powder which is free from celite.

2.1. Cellulase production of *C. indologenes LA4K*

Based on the rate of cellulase activity obtained in the previous stage, we could obtain information about the amount of time required to generate the highest rate of enzyme activity. Potential isolates were selected from those which had a relatively short logarithmic phase, required relatively short production time, and had a high cellulolytic enzyme activity. Those potential isolates were involved in the subsequent enzyme production and testing.

Cellulolytic enzyme production was carried out within the incubation period which has been proven to generate the highest rate of cellulolytic enzyme activity. This production stage started by regenerating the selected cellulolytic isolates.

10% of the inocula grown in a Nutrient Broth (NB) medium which had already produced approximately 108 colonies were taken and added to a 250 mL liquid medium composed of 2.5% agar-agar solid waste in a 500 mL Erlenmeyer flask. This solution was then incubated at 37°C at an agitation speed of 125 rpm. Harvesting was carried out on the sixth day, at the peak of cellulase
production. The culture was then centrifuged at 4°C for 10 minutes at a speed of 10,000 rpm. Centrifugation was performed to separate between sediment and supernatant which contained the crude enzyme. This supernatant would then be used in characterization testing.

2.2. Determining the optimum pH of cellulolytic enzyme activity

Determination of optimum pH was performed by reacting between enzyme and buffer (50 mM) with varied pH ranging from 3 to 10 before incubating the result for 30 minutes. After that, its cellulase activity was tested. Optimum pH was determined using citrate buffer (pH 3 and 4), citrate-phosphate buffer (pH 5 and 6), phosphate buffer (pH 7 and 8), and borate buffer (pH 9 and 10). A quantitative test of cellulase activity was performed by examining the enzyme’s ability to hydrolyze cellulose and produce simple sugar, which was measured using dinitrosalicylic acid (DNS) and CMC substrate [5,6].

2.3. Determining the optimum temperature of cellulolytic enzyme activity

Determination of optimum temperature was performed by reacting enzyme to a certain optimum pH at various reaction temperatures. The incubation temperatures applied in this study were 30, 40, 50, 60, 70, and 80°C, and the incubation period was 30 minutes. A quantitative test of cellulase activity was performed by examining the enzyme’s ability to hydrolyze cellulose and produce simple sugar, which was measured using dinitrosalicylic acid (DNS) and CMC substrate [5,6].

2.4. Determining cellulolytic enzyme activity on a particular substrate

This process was performed to determine the activity of a cellulolytic enzyme which was produced by a selected isolate on a particular substrate. According to Dashtban et al. (2010), a test of total cellulase activity can be performed by using Whatman filter paper Number 1 (1 x 3 cm strips) as substrate and applying the method proposed by Wood and Bhat (1988) in Dashtban et al. (2010). Meanwhile, to obtain the activity of individual cellulase, the test was adjusted to the type of cellulolytic enzyme under investigation. Endoglucanase activity was tested using CMC as a substrate [5,6,7]. Exoglucanase activity was tested using Avicel as a substrate. β-glucosidase activity was tested using cellobiose as substrate [7].

2.5. Influence of metal and EDTA addition on cellulolytic enzyme activity

This test was carried out by reacting enzyme at its optimum pH and temperature. The test involved the addition of several types of metal ions which consisted of monovalent metal cations Li⁺, K⁺, and Na⁺; bivalent metal cations Mg²⁺, Ca²⁺, Zn²⁺, Cu²⁺, and Mn²⁺; trivalent metal cations FeCl₃; and EDTA. Metal ion solution was added to the enzyme reaction mixture before the first incubation with final concentrations of 1 mmol/ml, 5 mmol/ml, and 10 mmol/ml. At the same time, positive control was carried out by not adding any metal ion solution to the enzyme reaction mixture. The activity of the resulting reaction was measured using CMC as a substrate. Besides that, we also analyzed the influence of anionic detergent (SDS), nonionic detergents (Triton X-100 and Tween 80), and an organic solvent (ethanol) on the activity of the crude enzyme from LA4K isolate.

3. Results and discussion

3.1. Influence of pH on the activity of cellulolytic enzyme of C. indologenes LA4K

One of the factors which may influence an enzyme’s activity is pH. The pH condition in the surrounding environment of an enzyme may alter the amino acid in the enzyme’s active site. This condition may prevent the enzyme’s active site from binding to the substrate. Each enzyme has a different range of optimum pH, depending on the enzyme type. The influence of pH on enzyme activity was observed at pH ranging from 3.0 to 10.0. The enzyme’s supernatant sample was reacted using a buffer solution at certain pH for 30 minutes before its activity was tested.

According to the results of the test performed on the cellulase obtained from LA4K isolate, the cellulase in a medium with 2.5% LIA substrate reached its optimum activity of 0.2254 U/mL at pH around 8. This finding is in line with that of a study by Li and Yu (2012) [8]. Li, who came from
Yuncheng, China, produced extracellular cellulase of a halotolerant isolate from *Bacillus* sp. which had an optimum pH of 8.0 at 60°C. The enzyme remained active and stable at the temperature ranging from 30 to 80°C and pH ranging from 7.0 to 9.0.

![Figure 1](image.png)

**Figure 1.** Influence of pH buffer on the activity of crude cellulolytic enzyme extracts derived from *C. indologenes* LA4K isolate

Enzyme activity is easily influenced by pH because of the ionic nature of carboxyl clusters and amino clusters. Therefore, any pH changes may cause the enzyme to become denatured and to lose its activity. The enzyme contains active clusters with both positive and negative charges. The enzyme would reach its optimum activity when these two opposing charges are in balance. During an acid state, it tends to have more positive charges, while during a basic state, it tends to have more negative charges, in which case its activity would decrease or even become inactive.

### 3.2. Influence of temperature on cellulolytic enzyme activity

Temperature plays an important role in the enzymatic reaction. When the temperature rises to a certain optimum point, the speed of the enzyme reaction will increase because of the kinetic energy increases. Such an increase in kinetic energy will speed up the vibrational, rotational, and translational motions of both enzyme and substrate. This condition increases the opportunity of reaction between enzyme and substrate. When the temperature is higher than the optimum temperature, protein conformation will change, thus inhibiting the reactive clusters. Such conformation change can cause the enzyme to become denatured. The optimum temperature of *C. indologenes* LA4K crude cellulase activity was determined by reacting the cellulase’s crude enzyme and substrate in an appropriate phosphate buffer, which was then incubated for 30 minutes.

Ladeira *et al.* (2015) [9] have produced thermophilic cellulase from *Bacillus* sp. SMIA-2 using sugar cane and corn as substrates which were incubated at the temperature of 50°C. The maximum activity of avicelase and CMCCase at optimum pH of 7.5 and 8.0 was 0.83 U/mL and 0.29 U/mL, respectively. The optimum temperature of avicelase and CMCCase was 70°C, and both of them remained 100% stable in spite of being heated at the temperature of 60°C for 1 hour. Determination of optimum temperature was performed by reacting the enzyme at pH 7 at various reaction temperatures, consisting of 30, 40, 50, 60, 70, and 80°C (figure 2). The cellulase activity of the enzyme reaction result was measured using the DNS method. The influence of temperature on optimum pH was demonstrated by an increase in enzyme activity. *C. indologenes* LA4K cellulase produced an optimum
activity of 0.3246 U/mL at 40°C (figure 2a). In order to determine the optimum incubation period of the cellulase’s crude enzyme and substrate, a test was performed at an interval of 30 minutes at its optimum temperature, i.e. 40°C. The optimum incubation period which was needed by C. indologenes LA4K cellulase to react with substrate was 30 minutes (figure 2b).

Figure 2. Influence of heating on enzyme activity: (a) determination of the optimum temperature of C. indologenes LA4K isolate cellulase activity at pH 8; (b) determination of the optimum incubation period of cellulase at the optimum temperature of 40°C; and (c) determination of heat resistance rate after heating the enzyme for 60 minutes at a certain temperature.
Determination of heat resistance rate of *C. indologenes* LA4K cellulolytic enzyme was carried out to identify the maximum temperature at which the enzyme would lose its activity. After being heated at a certain temperature for 60 minutes, the enzyme was then reacted with CMC substrate at its optimum pH and optimum temperature. The incubation period was found to influence the activity of reaction between *C. indologenes* LA4K crude enzyme and substrate at 40°C. After four hours of incubation, its activity was still 0.1733 U/mL or 55.97% of its initial activity, while it eventually lost its activity at the seventh hour (figure 2b).

Heating can influence the activity of the crude enzyme. When the crude enzyme of *C. indologenes* LA4K isolate was heated up to the temperature of 50°C, they still had an activity of 0.1372 U/mL, but this value dropped to 0.034 U/mL when the enzyme was further heated to the temperature of 70°C (figure 2c).

The crude cellulolytic enzyme of *C. indologenes* LA4K entirely lost its activity at the temperature of 80°C because they were denatured by the heat to such a degree that the conformation of the enzyme’s protein structure changed. This change damaged the enzyme’s active site, so the substrate cannot penetrate that active site (figure 2c). In terms of heat resistance, when *C. indologenes* LA4K cellulolytic enzyme was heated to the temperature of 50°C, it lost half of its initial activity. At 80°C, the enzyme entirely lost its activity.

### 3.3. Enzyme activity with various substrates

Even though it only had a low activity with xylan and FP substrates, the crude extract of cellulolytic enzyme from *C. indologenes* LA4K isolate demonstrated a relatively high activity with cellobiose and CMC substrates (figure 3). Avicel is crystalline cellulose whose test results show the presence of exo-1,4-β-glucanase enzyme activity which cut the end of oligosaccharide chain and converted it into cellobiose, i.e. two glucose molecules which are linked by β-1,4-glycosidic bond. The activity of the crude enzyme extract of *C. indologenes* LA4K isolate with LIA substrate was almost the same with that with Avicel substrate.

![Figure 3](image-url) Activities of *Indologenes* LA4K enzymes tested with various types of substrates

### 3.4. Influence of metal and EDTA addition on cellulase activity

Several metal ions, namely Mg²⁺, Ca²⁺, and Zn²⁺, were found to significantly increase the activities of both enzymes (figure 4). The influence of divalent metals Mg²⁺, Ca²⁺, Zn²⁺ on enzyme activity increases significantly as the concentration of those metal ions increases. Other divalent metals such as Cu²⁺ and Mn²⁺ tend to inhibit enzyme activity. Such inhibition is possible because metals such as...
Cu\(^{2+}\) and Mn\(^{2+}\) are generally toxic in nature. At certain concentrations, monovalent metals such as K\(^+\), Li\(^+\), and Na\(^+\) only had little influence on enzyme activity. 

*Clostridium indologenes* LA4K cellulase demonstrated an increase in cellulase activity with divalent ions (Mg\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\)) and monovalent ions (K\(^+\) and Na\(^+\)) (figure 4). An addition of trivalent ion Fe\(^{3+}\) to *Clostridium indologenes* LA4K cellulase was found to inhibit its activity.

Several studies show that the presence of metal ions can influence enzyme activity [8,10,11]. Divalent metals such as Ca\(^{2+}\) and Mg\(^{2+}\) have been known to increase enzyme activity [11]. The presence of these cations is important as they have a great role in protecting enzymes, strengthening their active site, and shaping their conformation. An increase in cellulase activity was greatly influenced by the presence of metal ions Mg\(^{2+}\) and Ca\(^{2+}\), but inhibited by EDTA. This shows that the enzyme belongs to a group called metalloenzyme. EDTA is known as a chelating agent and has an inhibitory effect [8,10,12].

![Figure 4. Influence of metal ions on *Clostridium indologenes* LA4K cellulase activity](image)

**Figure 4.** Influence of metal ions on *Clostridium indologenes* LA4K cellulase activity

### 3.5. Influence of organic solvent and detergent on enzyme activity

Our test results show that organic solvent ethanol and a number of anionic and nonionic detergents had some influence on the enzyme activity LA4K isolates. Addition of organic solvent ethanol to crude supernatant enzyme caused the enzyme to become denatured, thus decreasing the cellulase activity of *Clostridium indologenes* LA4K isolates to 78.67\% and 85.47\%, respectively (figure 5). This happened because ethanol is an organic solvent with an extremely toxic characteristic [12]. The presence of anionic detergent SDS and nonionic detergents Triton X-100 and Tween 80 increased the cellulase activity of both isolates. The presence of anionic detergent SDS in the crude cellulase of *Clostridium indologenes* LA4K increased the isolates’ activity to 112.76\% and 105\%, respectively. Nonionic detergents Triton X-100 and Tween 80 detergents increased the activity of *Clostridium indologenes* LA4K cellulase to 130\% and 110.9 \%, respectively.

One of the 19 isolates which were isolated by Trivedi *et al.* (2011) [12], namely *Bacillus* NT1, has the potential for generating extracellular alkaline cellulase and is tolerant to several types of organic solvents, such as acetone, methanol, and benzene. This extracellular *Bacillus* NT1 has an optimum activity at pH 11 and at the temperature of 45°C. The enzyme activity after an addition of divalent and monovalent metals is presented as Co\(^{2+}\) > Fe\(^{2+}\) > Cu\(^{2+}\) > K\(^+\) > Na\(^+\).

In research by Trivedi *et al.* (2011) [12], the activity of cellulase derived from *Bacillus aquimaris* increased two times with an addition of Fe\(^{2+}\) ion, while an addition of alkaline metal ion did not
influence its activity at all, meaning that it remained 100%. Meanwhile, addition of anionic detergent SDS and nonionic detergents Tween 80 and Triton X-100 increased the cellulase activity of *Bacillus aquimaris*. SDS is an anionic organosulfur which has a 12-carbon tail attached to sulfate clusters and is commonly used as a material for producing detergent. Tween 80 and Triton X-100 are nonionic surfactants which are commonly used as an emulsifier [13, 14].

Conversion of cellulose into fermenting sugar by means of enzymatic hydrolysis is one of the most expensive stages in bioethanol production in terms of cellulase activity and quantity required in the process. Additives such as nonionic surfactants have a positive influence on enzymatic hydrolysis of cellulose in that they increase the enzymatic conversion of corn and straw waste into bioethanol [13]. Surfactants or surface active agents are amphiphilic compounds which have hydrophilic heads and hydrophobic tails. Nonionic surfactants have a significant influence on enzymatic hydrolysis of cellulose at both low and high concentrations. Several factors contribute to this: (1) surfactants can act as an enzyme stabilizer and prevent denaturation; and (2) surfactants influences substrate structure, meaning that surfactants can modify a substrate, thus enabling enzyme to access substrate more effectively. In addition to those two factors, surfactants may also trigger interaction between enzyme and substrate, thus enabling the substrate to absorb enzyme in a more productive way [13].

**Figure 5.** Influence of organic solvent and detergents on the activity of *C. indologenes* LA4K crude cellulase

Addition of nonionic surfactants (Tween 40, Tween 60, Tween 80, and Triton X-100) and anionic detergent (SDS), all at a concentration of 0.1%, increases the activity of cellulolytic enzyme derived from *Bacillus vallismortis* RG-07 bacterium [14]. Nevertheless, there are also studies which do not support the idea that surfactants have a positive influence on enzyme activity. Such studies conclude that surfactant addition does not have any influence on enzyme activity, while several others even mentioned that this treatment actually decreases enzyme activity, such as the ones carried out by Li and Yu (2012) [8] and Ladeira et al. (2015) [9]. In their research, Li and Yu (2012) [8] added anionic detergent SDS to the cellulase which they had produced from *Bacillus* sp. L1, but its enzyme activity decreased to 58.9%. The same also happened when they added nonionic surfactants Triton X-100, Tween 80, and Tween 20, which slightly reduced the enzyme activity to 91.5%. Meanwhile, in their research, Ladeira et al. (2015) [9] added two different surfactants to the cellulase which they had produced from *Bacillus* sp. SMIA-2, namely anionic detergent SDS and nonionic detergent Triton X-100. In the first treatment, the enzyme activity was stable, but in the second treatment, the enzyme activity was inhibited.
4. Conclusion
Solid waste from agar-agar industry can be utilized as one of the sources of carbon in the growth media and production media of cellulolytic enzymes. The cellulolytic enzyme of C.indologenes LA4K was incubated for six days using 2.5% agar-agar industry solid waste as medium and showed an activity of 0.3726 U/mL. Based on the heat resistance test, C. indologenes LA4K cellulase lost more than half of its initial activity when heated to 50°C. Furthermore, the enzyme entirely lost its activity when heated to 80°C. This enzyme was found to reach its optimum activity at pH 7 to 9, and this characteristic shows that this cellulolytic enzyme can be utilized in pulp and paper industries.

Acknowledgment
This research was supported/partially supported by Marine and Fisheries Product Processing and Biotechnology, Marine and Fisheries Ministry, Jakarta, Indonesia and CV Agar Sari Malang. We have to express out appreciation to the Prof. Ekowati Chasanah for sharing their pearls of wisdom with us during the course of this research.

References
[1] Kumar S, Gupta R, Kumar G, Sahoo D and Kuhad RC 2013 Bioethanol production from Gracilaria verrucosa, a red alga, in a biorefinery approach Biorest Technol. 135 150–156
[2] Marinho-Soriano E, Silva TS and Moreira WSC 2001 Seasonal variation in the biomass and agar yield from Gracilaria cervicornis and Hydropuntia cornea from Brazil Biorest. Technol. 77 115-120
[3] Marinho-Soriano E and Bourret E 2005 Polysaccharides from the red seaweed Gracilaria dura (Gracilariales, Rhodophyta) Biorest Technol. 96 379-382
[4] Munifah, Sunarti TC, Irianto HE and Meryandini A 2015 Biodiversity Of Cellulolytic Bacteria Isolated From The Solid Wastes Of Agar Seaweed Processing Industry. Biosci. Biotech. Res. Asia 12(3) 1957-1964
[5] Eveleigh DE, Mandels M, Andreotti R and Roche C 2009 Measurement of saccharifying cellulase. Biotechnol Biofuels. 2 21-27
[6] Kim HT, Lee S, Kim KH and Choi IG 2012 The complete enzymatic saccharification of agarose and its application to simultaneous saccharification and fermentation of agarose for ethanol production. Biorest Technol. 107 301–306
[7] Dashtban M, Maki M, Leung KT, Mao C and Qin W 2010 Cellulase activities in biomass conversion: measurement methods and comparison Critic Revi in Biotechnol. 1 1-8.
[8] Li X and Yu HY 2012 Purification and characterization of an organic-solvent-tolerant cellulase from a halotolerant isolate, Bacillus sp. L1. J Ind Microb Biotechnol 39(8) 1117–1124
[9] Ladeira SA, Cruz E, Delatorre AB, Barbosa JB and Martins, MLL 2015 Cellulase production by thermophilic Bacillus sp. SMIA-2 and its detergent compatibility. Electron J Biotechnol. 18(2):110–115
[10] Trivedi N, Gupta V, Kumar M, Kumari P, Reddy CRK and Jha B 2011a An alkali-halotolerant cellulase from Bacillus flexus isolated from green seaweed Ulva lactuca Carbo Polymers 83 891–897
[11] Kotchoni SO, Gachomo EW, Omamwbe BO and Shonukan OO 2006 Purification and Biochemical characterization of carboxymethyl cellulase (cmmc) from a catabolite repression insensitive mutant of Bacillus pumilus Int. J. Agri. Biol. 8 (2) 286–292
[12] Trivedi N, Gupta V, Kumar M, Kumari P, Reddy CRK and Jha, B 2011b Solvent tolerant marine bacterium Bacillus aquimaris secreting organic solvent stable alkaline cellulase Chemosphere 83(5) 706–712
[13] Kristensen JB, Felby C and Jørgensen H 2009 Yield-determining factors in high-solids enzymatic hydrolysis of Lignocellulose Biotechnol Biofuels 2 11
[14] Gaur R. and Tiwari S 2015 Isolation, production, purification, and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnol.*, 15(1) 19

[15] Marinho-Soriano E 2001 Agar polysaccharides from *Gracilaria* species Rhodophyta (Gracilariaceae) *J Biotechnol.* 89 81-84