WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 8, 1251-1260. Research Article ISSN 2277–7105

PRODUCTION & PURIFICATION OF A LOW MOLECULAR WEIGHT EXTRACELLULAR TANNASE FROM *ASPERGILLUS* TPF-4

Sunny Dhiman*, Gunjan Mukherjee and Anu Kumar

University Institute of Biotechnology, Chandigarh University, Mohali, Punjab, INDIA.

Article Received on
25 May 2017,

Revised on 14 June 2017,
Accepted on 05 July 2017

DOI: 10.20959/wjpr20178-8970

***Corresponding Author**
Sunny Dhiman
University Institute of Biotechnology,
Chandigarh University,
Mohali, Punjab, INDIA.

ABSTRACT

In the present investigation, tannase production potential of *Aspergillus* spp. designated as *Aspergillus* TPF-4 was assessed in submerged culture utilizing tannic acid as substrate. Assessment of enzyme activity revealed that *Aspergillus* TPF-4 strain possessed the proficiency of producing a higher titer of extracellular tannase. The potential of ammonium sulphate precipitation & anion exchange chromatography for tannase purification was evaluated. The crude enzyme exhibited maximum activity at 20% saturation concentration of ammonium sulphate. Precipitation of tannase protein was achieved at 50 % saturation concentration of ammonium sulphate. The concentrated enzyme flaunted a purification fold of 28.5 with 11.40% yield upon purification by anion-exchange chromatography using DEAE-cellulose column. Purified tannase protein unveiled a single band with an apparent molecular weight of approximately 35.0kDa upon SDS-PAGE analysis. A single protein band verifies its homogeneity and complete purification.

KEYWORDS: *Aspergillus* TPF-4, Tannase, Purification, Anion exchange chromatography, Submerged fermentation, SDS-PAGE.

1 INTRODUCTION

Tannin acyl hydrolase (E.C.3.1.1.20) universally known as tannase is an inducible enzyme that has been widely utilized in the biotransformation of hydrolysable tannins to simple phenolic molecules like gallic acid. Tannases precisely act upon ester & depside linkages in hydrolysable tannins particularly the gallotannins thereafter giving off gallic acid and glucose molecule.[1] Tannins belong to phenolics, one of the major classes of plant secondary

metabolites that serve as a defense machinery against attack by herbivores. Tannins universally exist in angiosperms, gymnosperms as well as pteridophytes. Tannins predominantly accumulate in plant parts such as bark, roots, fruits & leaves.[2] Microbes put themselves ahead of chemical technology as an ideal source of industrial enzymes due to their diversified nature, ease of cultivation, better control over process parameters and ability to undergo genetic modification. Filamentous fungi belonging to *Aspergilli* genera have been documented as the one of the most potent tannase producer worldwide.[3] Submerged fermentation has been the most preferred method for tannase production worldwide. According to an estimate, worldwide market for industrial enzymes was evaluated around $4.2 billion in 2014 and is anticipated to hit approximately $6.2 billion mark globally at a compound annual growth rate of around 7 % over the period from 2015 to 2020 (2015 a,b: Industrial Enzyme Market).Globally the commercial manufacturing of tannase is being undertaken by JFC GmbH (Germany), Wako Pure Chemical Industries, Ltd. (Japan), Kikkoman (Japan), Novo Nordisk (Denmark), Sigma Aldrich Co. (USA),ASA special enzyme GmbH (Germany), Biocon (India), Amano (Japan), Julich (Germany), etc.in different forms with varying catalytic units.[4] Tannase has a vast range of applications in various industrial bioprocesses ranging from food, feed to chemical as well as pharma sector. The most significant application of tannase is the gallic acid production from hydrolysable tannins.[5] Gallic acid is a versatile precursor for the manufacture of a variety of chemicals used in food and pharmaceutical industries. Over the years, gallic acid has emerged as a highly valuable molecule credited to its worldwide use and demand as an antioxidant, antiviral, radio protective agent, anticancer & antitumor agent. Gallic acid is utilized worldwide as a precursor for manufacturing of trimethoprim, a broad spectrum antibacterial agent which is a bacteriostat, since it inhibits folic acid metabolism in pathogenic bacteria.[6] Gallic acid is substantially utilized in the preparation of lubricants, adhesives, wide range of cosmetics, significant number of hair products as well as in the preparation of pyrogallol; one of the most valuable gallic acid intermediate.[7] Pyrogallol itself is a highly valuable product which is substantially utilized in hair colouring, staining of fur and in the manufacturing of photographic plate developing agent.[8] Gallic acid is extensively utilized in the preparation of food preservative propyl gallate. Furthermore its application as a crop protection agent in agriculture sector marks it as an extremely valuable molecule. The advancements in molecular tools and techniques have enabled a better understanding of tannase structure, underlying mechanism of its action as well as the more precise understanding of various process parameters governing tannase production.Apart from gallic acid production tannase

is substantially utilized in instant tea preparation, juice clarification, detanning of food & feed and in the eviction of haze formation in fruit juices.[9] [10] [11] Furthermore tannase based treatment of toxic tannery effluent offers a cheaper and much reliable way for bioremediation of toxic tannery effluent.This tremendous biocatalytic potential of tannase is attributed to their higher stability and feasibility. Purification of tannase has been attempted earlier using one or the other purification protocols. Downstream processing essentially involves processes employed to lessen and evict the impurities from the end product. Various techniques viz high performance liquid chromatography, affinity chromatography, electrophoresis and ultrafiltration etc. are utilized worldwide for end product purification.[12] However, as a matter of fact these purification techniques give relatively lower yields upon purification. Furthermore these purification techniques are quite expensive. Since commercial applications don't demand extremely high degree purity of enzymes; this necessitates the utilization of rapid purification protocols to keep the process economical.[13] Thus on the grounds of this it was considered meaningful to employ a simple and relatively cheaper purification protocol to purify the crude tannase enzyme. Keeping these facts in view, the present investigation reports the production of extracellular tannase from *Aspergillus* TPF-4 in submerged culture conditions. Further the potential of ammonium sulphate precipitation & anion exchange chromatography for partial & complete purification of crude tannase was investigated.

2. MATERIAL AND METHODS

2.1 Microorganism and inoculum preparation

Microorganism for extracellular tannase production was isolated from soil sample of Gharuan, Punjab; identified as *Aspergillus* spp. using manual about the genus *Aspergilli*[14][15] and designated as *Aspergillus* TPF-4. Inoculation of *Aspergillus* TPF-4 spores was done on PDA (Potato Dextrose Agar) plates, grown at 25^{o}C for 6 days and storage was done at 4°C. Inoculum preparation of completely sporulated culture was executed with the addition of autoclave sterilized distilled water (20ml) having 0.01% Tween 80.[16]

2.2 Enzyme production in submerged conditions

Enzyme production medium was prepared by Potato Dextrose Broth 2.4g; yeast extract 1.0g. Filter sterilized substrate tannic acid 2.5g was added to the autoclave sterilized media. Spore suspension (1.62 X 10^5 spores) of *Aspergillus* TPF-4 was added to 100ml of production medium in 250ml Erlenmeyer flasks. Culture was grown at 30^{o}C up to 120 h at 100 rpm in an incubator shaker. Removal of cell biomass was done by filtration using Whatman No. 1 filter

paper. Supernatant (crude enzyme) was assayed to evaluate the tannase activity at constant intervals of 24 hours.

2.3 Tannase Assay

Assessment of enzyme activity was done by using method proposed by Sharma *et al.*, 2000.[17] The reaction mixture was incubated at 40^{o}C for 5 minutes and optical density was recorded at 520nm using a spectrophotometer (Shimazdu UV-160A, Japan). Enzyme activity was represented in international units. One unit of enzyme activity was described as the amount of enzyme needed to liberate one micromole of gallic acid per minute under standard assay conditions.

2.4 Precipitation and purification of enzyme

All the exercises pertaining to purification of tannase were carried out at 4ºC in 0.05 M citrate buffer (pH- 5.5).The crude extracellular tannase was precipitated with ammonium sulphate precipitation as an initial step of purification. Precipitated tannase protein was collected by centrifugation at 15,000g for 30 mins at 4°C. The precipitates thus collected were resuspended in 10ml of 0.05 M citrate buffer (pH- 5.5).The concentrated enzyme was further purified by using anion exchange chromatography using DEAE-cellulose column (Aristogene PCA 003). The concentrated enzyme sample (129.17 U/mg) of ammonium sulphate precipitation was loaded on to the column previously equilibrated with citrate buffer (0.05M, pH-5.5). Protein fractions (1.0ml) were eluted at flow rate of 0.5ml/min initially with citrate buffer (0.05M, pH-5.5) and then sequentially with citrate buffer(pH 5.5) having different ionic strengths (0.3M, 0.5M, 0.7M, 1.0M). The same citrate buffer (0.05M, pH 5.5) was used to wash the column to discharge the bound protein. Ten protein fractions (1.0ml) were pooled. Dialysis of the pooled protein fractions was done against water and freeze drying method was followed to concentrate them and storage was commenced at -20^{o}C.[18] The pooled fractions were assayed for tannase activity.

2.5 Protein quantification

Protein quantification was accomplished by Bradford (1976) method using BSA as standard.[19] The reaction mixture consisted of 200µL of protein sample to which 2 mL of Bradford working reagent was added and contents were mixed by vortexing. The absorbance of reaction mixture was read at 595 nm after 5 min. Standard curve was prepared using 20-200 µg/mL of bovine serum albumin.

2.6 SDS-PAGE analysis of tannase

Purified tannase protein was analysed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to determine its purification profile & molecular weightaccording to the method described by Laemmli (1970).[20] Vertical slab gel was used to run the electrophoresis. The separating gel consisted of 8 % acrylamide & the stacking gel consisted of 4% acrylamide. The electrophoresis was commenced at 100V till the bromo-phenol blue tracking dye approached the alternate edge of the gel. Coomassie blue solution was utilized to detect the separated protein band. Molecular weight determination of the purified tannase protein was done using a standard protein marker purchased from Aristogene Biosciences Pvt. Ltd. Bangalore (India).

3. RESULTS AND DISCUSSIONS

3.1 Microorganism for tannase production

Microorganism for extracellular tannase production was explored from soil sample of Gharuan, Punjab and identified as *Aspergillus* spp. using manual about the genus *Aspergilli*[14] [15] and designated as *Aspergillus* TPF-4(Fig. 1)

Fig 1: Growth of *Aspergillus*TPF-4 on potato dextrose agar plate.

3.2 Tannse precipitation and purification

Tannase from *Aspergillus* TPF-4 was purified using ammonium sulphate precipitation & anion exchange chromatography through DEAE-cellulose column**.** The activity profile of tannase was determined after precipitation with different concentrations of ammonium sulphate. Maximum activity (45.75 U/mg) was obtained at 20% saturation concentration of ammonium sulphate. Tannase protein was precipitated upto 50 % saturation(Table 1.1). The

ammonium sulphate precipitated enzyme was loaded on to the DEAE-cellulose column. Rajkumar and Nandy (1983) documented 69% yield of tannase protein upon ammonium sulphate precipitation. [21] The ammonium sulphate precipitated enzyme displayed 28.55 fold purification with anion-exchange chromatography using DEAE-cellulose column. Similar results were documented by Hamdy, 2008, with a purification fold of 27 for tannase protein from *F. Subglutinans* with 65% ammonium sulphate optimum for precipitation thereafter ion exchange chromatography.[1] Purification fold of 19.5& 19.4 were documented by Chhokar *et al.*, 2009 & Mahendran *et al.*, 2006, upon purification of tannase using DEAE-cellulose column chromatography.[22] [23] Enzyme activity was recorded only in the fractions viz.3, 4, 5, 6, 7, 8, 9 and 10 with maximum activity in the fraction number 5(Fig.2). These fractions were pooled together and were assessed for purity by SDS PAGE. The detailed purification profile of tannase has been summarized in Table (1.2).

Table 1.1: Activity profile of Ammonium sulphate precipitation.

Ammonium sulphate (%)	Specific activity (µmol/min/mg)
0-10	26.22
10-20	45.75
20-30	37.74
30-40	18.82
40-50	4.00
50-60	-
60-70	-
70-80	-

Table 1.2: Purification profile of tannase from *Aspergillus* TPF-4.

Purification steps	Volume (mL)	Total protein (mg)	Specific activity (µmoles/min/mg)	Total activity (U)	Yield (%)	Fold Purification
Crude Enzyme	300	154.8	15.05	2329.7	100	1
Ammonium sulphate Precipitation	6	6.21	129.17	775.02	33.26	8.58
DEAE-Cellulose Chromatography	6	0.618	429.70	265.55	11.40	28.55

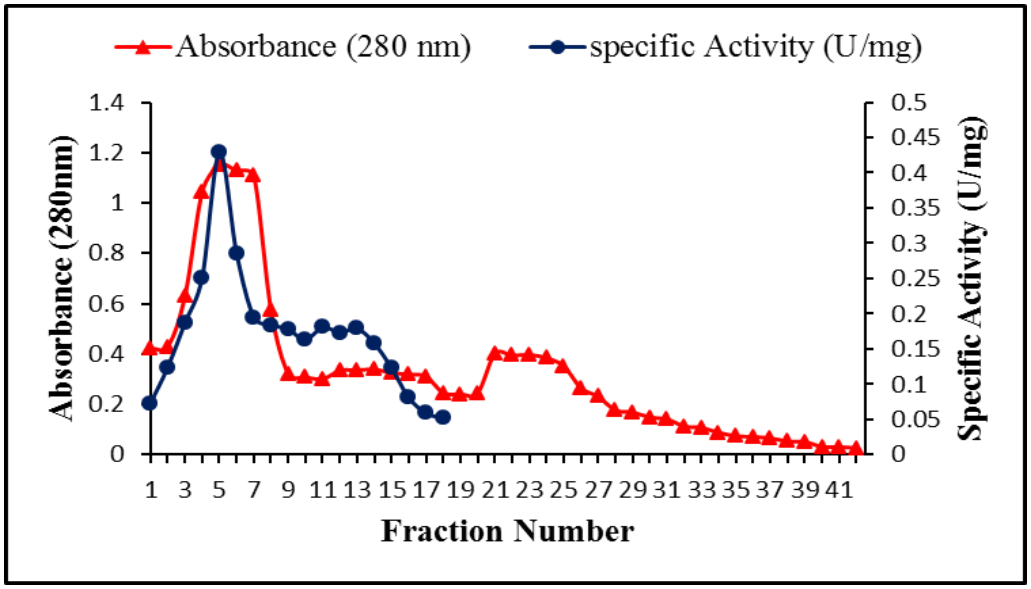

Fig 2: Activity profile of purified tannase from *Aspergillus* TPF-4.

3.3 Molecular weight determination

Apparent molecular weight of tannase as determined using SDS-PAGE analysis was approximately 35kDa. A single protein band verifies its homogeneity and complete purification(Fig. 3). Tannase from *A. niger* ATCC 16620 has a single monomeric unit of 168 kDa (Sabu *et al*., 2005).[24] The tannases with a molecular weight of 45 kDa from *Paecilomyces variotii*,[23] 59 kDa from *Selenomonas ruminantium*,[25] 63 kDa from *Aspergillus oryzae*,[26] 101 kDa from *Aspergillus awamori* MTCC 9299,[22] 180 kDa from *Aspergillus tamarii*,[16] 310 kDa for tannase from *Penicillium variable*,[27] 320 kDa from *Arxula adeninivorans*,[28] etc. have been documented in earlier studies.

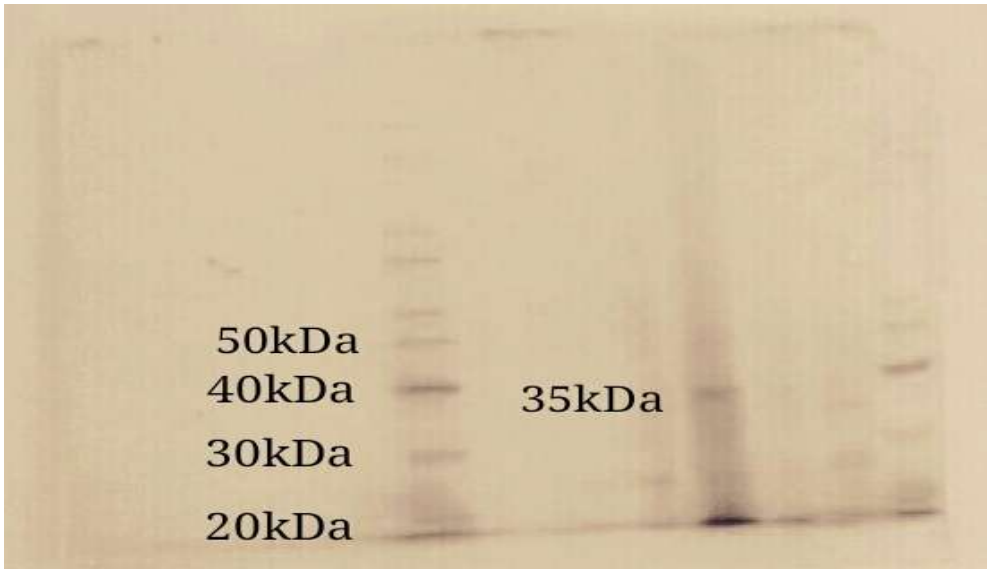

Fig 3: SDS-PAGE of purified tannase of *Aspergillus*TPF-4.

CONCLUSION

The present work entitltled "Production & purification of a low molecular weight extracellular tannase from *Aspergillus* TPF-4"was taken up with a view of utilising *Aspergillus* spp. as a microbial source for the extracellular production of tannase which has the capability to hydrolyze tannic acid to gallic acid.From our study we concluded that this strain was capable of producing a higher titer of extracellular tannase. Precipitation of tannase protein was achieved at 50 % saturation concentration of ammonium sulphate. The concentrated enzyme flaunted a purification fold of 28.55 with 11.40% yield upon purification by anion-exchange chromatography using DEAE-cellulose column. These results clearly demonstrate that crude tannase protein was significantly purified. Molecular weight of 35kDa indicates this enzyme to be one among the smallest tannase of fungal origin. A single protein band upon SDS-PAGE analysis confirms its homogeneity and complete purification. Purified tannase protein with such a low molecular weight may prove quite promising for utilization in various industrial bioprocessing applications requiring low molecular weight enzymes.Since the enzyme tannase has a broad range of applications; there is always an opportunity for a tannase with better & improved characteristics.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest in relation to this research article.

REFERENCES

1. Hamdy H S. Purification and characterisation of a newly isolated stable long-life tannase produced by *F. subglutinans*. J. Pharm. Innov, 2008; 3: 142–151.
2. Scalbert A.. Antimicrobial properties of tannins. Phytochem, 1991; 30: 3857-3883.
3. Banerjee D, Mondal KC, Pati BR. Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. J. Basic Microbiol, 2001; 41: 313-318.
4. Aguilar CN, Rodriguez R, Gutierrez-Sanchez G, Augur C, Favela-Torres E, Prado-Barragan LA, Ramirez-Coronel A, Contreras-Esquivel JC. Microbial Tannases: Advances and Perspectives. Appl. Microbiol. Biotechnol, 2007; 76: 47–59.
5. Kar B, Banerjee R, Bhattacharyya BC. Optimization of physicochemical parameters of gallic acid production by evolutionary operation-factorial design technique. Pro. Biochem, 2002; 37: 1395-1401.

6. Mukherjee G, Banerjee R. Production of gallic acid. Biotechnological routes (Part 1). Chimica Oggi, 2003; 21: 59-62.
7. Yu XW, Li YQ. Kinetics and thermodynamics of synthesis of propyl gallate by mycelium-bound tannase from *Aspergillus niger* in organic solvent. J. Mol. Catal. B: Enzym, 2006, 40: 44–50.
8. Zeida M, Wieser M, Yoshida T, Sugio T, Nagasawa T. Purification and characterization of gallic acid decarboxylase from Pantoea agglomerans T71. Appl. Environ. Microbiol, 1998, 64: 4743–4747.
9. Belmares, R, Contreras-Esquivel JC, Rodriguez-Herrera R, Coronel AR, Aguilar CN. Microbial production of tannase: an enzyme with potential use in food industry. Lebensmittel-Wissenschaft Technologie, 2004; 37: 857-64.
10. Mohapatra PKD, Maity C, Rao RS, Pati BR, Mondal KC. Optimization of submerged culture conditions by Taguchi DOE methodology. Food Res. Int, 2009; 42: 430–435.
11. Goel G, Puniya AK, Aguilar CN, Singh, K. Interaction of gut microflora with tannins in feeds. Naturwissenchaften, 2005; 92: 947- 503.
12. Mukherjee G, Banerjee R. Effects of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. World. J. Microbiol. Biotechnol, 2006, 22: 207-212.
13. Battestin V, Macedo G, Pastore P. Optimizing the fermentation broth for tannase production by a new isolated strain *Paecilomyces variotii*. *J. Technol*, 2005; 118: S49.
14. McClenny N. Laboratory detection and identification of aspergillus species by microscopic observation and culture: the traditional approach. J. Med. & Vet. Mycol, 2005; (Suppl. 1): 43: S125-S128.
15. Diba K, Kordbacheh P, Mirhendi SH, Rezaie S, Mahmoudi M. Identification of *Aspergillus* species using morphological characteristics. Pak. J. Med. Sci, 2007; 23(6): 867-872.
16. Costa AM, Kadowaki MK, Minozzo MC, Souza CGM, Boer CG, Bracht A, Peralta RM. Production, purification and characterization of tannase from *Aspergillus tamarii*. Afri. J. Biotechnol, 2012; 11: 391-398.
17. Sharma S, Bhat TK, Dawra RK. A spectrophotometric method for assay of tannase using Rhodonine. Anal. Biochem, 2000; 278: 85-89.
18. Lal D, Divya Shrivastava D, Verma H N, Gardne J J. Production of Tannin Acyl Hydrolase (E.C. 3.1.1.20) from *Aspergillus niger* isolated from bark of *Acacia nilotica.* J. Microbiol Biotechnol Res, 2012; 4: 566-572.

19. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem, 1976; 72: 248-254.
20. Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970; 227: 680–685.
21. Rajakumar GS, Nandy SC. Isolation purification and some properties of *Penicillium chrysogenum* tannase. *Ap. Env. Microbiol*, 1983; 46(2): 525–527.
22. Chhokar V, Sangwan M, Beniwal V, Nehra K, Nehra KS. Effect of additives on the activity of tannase from *Aspergillus awamori* MTCC9299. Ap. Biochem. Biotechnol, 2009; 9: 8813-8817.
23. Mahendran B, Raman N, Kim D J. Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. Ap. Microbiol. Biotechnol, 2006; 70(4): 444–450.
24. Sabu A, Kiran GS, Pandey A. Purification and characterization of tannin acyl hydrolase from *A. niger* ATCC 16620. Food Technol. Biotechnol, 2005; 43: 133-138.
25. Skene IK, Brooker JD.. Characterization of tannin acyl hydrolase in the ruminal bacterium *Selenomonas ruminantium*. Anaerobe, 1995, 1: 321-327.
26. Hatamoto O, Watarai T, Kikuchi M, Mizusawa K, Sekine H. Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*. Gene, 2005, 175: 215-221.
27. Sharma S, Agarwal L, Saxena RK. Purification, immobilization and characterization of tannase from *Penicillium variable*. Bioreso. Technol, 2008; 99: 2544–2551.
28. Boer E, Bode R, Mock HP, Piontek M, Kunze G. Atan Ip-an extracellular tannase from the dimorphic yeast *Arxula adeninivorans:* molecular cloning of ATAN1 gene and characterization of the recombinant enzyme. Yeast, 2009; 26: 323-337.

(2015, a) India industrial enzyme market: forecast and opportunities 2020. http://www.pharmaion.com/report/india-industrial-enzymes-market-forecast-and-opportunities-2020/10.html.

(2015, b) Industrial enzymes market by type (carbohydrases, proteases, non-starch polysaccharides & others), application (food & beverage, cleaning agents, animal feed & others), brands & by region—global trends and forecasts to 2020. www.bccresearch.com. http://www.marketsandmarkets.com/Market-Reports/industrial-enzymes-market-237327836.html.