

Study on optimization of extra-cellular polysaccharides to *Cordyceps sinensis* by submerged fermentation

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ABSTRACT

Dongchongxiachao is for short chongchao; polysaccharide of chongchao is gaze at physiology activities of antitumor and dropping blood sugar, however crude chongchao is few and has strict growing condition, recently plenty research is displaced crude chongchao by fermenting *Cordyceps sinensis*. On this purpose of study is consist in cultivating *Cordyceps sinensis* by submerged culture and analyzing relationship between ingredients of medium and a yield of polysaccharide of chongchao, expected to obtain maximum yield using optimum compose of medium by response surface methodology. The result is discovered that fermentation of *Cordyceps sinensis* had maximum biomass and extra-cellular polysaccharides contents are 1.02 g/dL and 1.082 mg/mL at the sixth days, and also was analyzed molecular weight of extra-polysaccharides at the fermentation later stage and earlier stage, the result show that no matter what biomass and extra-cellular polysaccharides of *Cordyceps sinensis* using corn steep powder medium is better than yeast powder medium. At the aspect of compared fermentation *Cordyceps sinensis* by different carbon to nitrogen ratio, although more nitrogen ratio was added and more extra-cellular polysaccharides of *Cordyceps sinensis* measured, but biomass of *Cordyceps sinensis* was restrained by being added corn steep powder ratio over 3 % and then observed the trend of extra-cellular polysaccharides during the ten fermentation using as 3 % corn steep powder and 2.5 % glucose added at medium, it was discovered that the maximum extra-cellular polysaccharides contents was measured at the fourth days during the fermentation was 1.17 mg/mL, it seems that extra-cellular polysaccharides not increased direct proportion. When it was fixed the nitrogen ratio at 1 %, more carbon source added and more extra-cellular polysaccharides contents was on the rise. It was restrained the extra-cellular polysaccharides contents when nitrogen ratio was 3 % and carbon source was 5 %. At the conferred extra-cellular polysaccharides content during the earlier experiment of response surface methodology, five kind of carbon source was chose sucrose as the carbon source makes the content of extra-cellular polysaccharides obvious excellent than the other ones although biomass content was not the best. It used as different ratio of nitrogen source (between the 0.25 %-1.5 %), it was comparatively had much content of extra-cellular polysaccharides using 0.5 % corn steep powder. At the different initial pH value, it was comparatively had much content of extra-cellular polysaccharides at pH 4.5. It was depended on the result of the earlier experiment of response surface methodology, using the 24-1 portion factor experiment designed to confer interaction between the extra-cellular polysaccharides content and each factor, it discovered that sucrose, corn steep powder and salt had positive effect on content of extra-cellular polysaccharides, however the initial pH value had negative effect but in opposition to the other factor was not outstanding. It used as method of steepest ascent path to close in the range of maximum content of extra-cellular polysaccharides and discovered that used as 5.42 % sucrose, 0.564 % corn steep powder and 0.65 % salt to compose the medium had the maximum content of extra-cellular polysaccharides was 11.23 mg/mL, it was used method of central composite experiment design to get the most suitable medium composition, sucrose was 5.846 %, corn steep was 0.561 % and 0.5344 % (NH₄)₂HPO₄ and 0.1336 % KH₂PO₄ as salt source, for this reason used the ratio of the most suitable medium composition to ferment the *Cordyceps sinensis* in the fermentor and had the maximum content of biomass at the fifth days during fermentation was 1.94 g/dL, and the maximum content of extra-cellular polysaccharides was 13.46 mg/mL, and seemed that did not control the pH in the fermentor was benefit to a yield of extra-cellular polysaccharides than did during the fermentation.

Keywords : *Cordyceps sinensis* ; analysis of extra-cellular polysaccharides ; response surface methodology

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REFERENCES

- 孔祥環、蔣保季、王惠琴、尹學鈞、唐玉萍、馬忠杰、沈家琴、張宏傳與肖中新。1995。發酵培育冬蟲夏草毒理研究 II、致突變性研究。首都醫學院學報 16(4):256-258。
- 王西華、陳志昇、黃雅惠與許瑞祥。1999。傳統保健食品 冬蟲夏草。生物產業 10(1):19-27。
- 水野卓與川合正允。1997。菇類的化學、生化學。國立編譯館，台北。pp40。
- 任一平、黃百芬與陳青俊。1996。應用高效液相色譜法測定香菇多糖。食品與發酵工程 5:31-35。
- 沈曉云、李兆蘭與田軍。1998。冬蟲夏草與蟲草菌絲有效成份分析比較。山西大學學報 21(1):80-85。
- 李玲玲、王正怡與蘇慶華。1995。利用流動細胞分析儀測植生蟲草抗腫瘤多醣體(PN-2)對小白鼠巨噬細胞吞噬能力及輔助 T 淋巴細胞活性之影響。北醫學報 23(1):11-19。
- 李昌憲、紅哲穎與熊光濱。1992。利用回應曲面法進行以 *Streptococcus faecalis* 生產酪胺酸脫羧酶之培養基最適化研究。中國農業化學會誌 30(2):264-272。
- 李繡鈴、周正俊與吳淳美。1993。利用反應曲面法尋求 *Sporobolomyces odorus* 產生 -decalactone 之最適條件。中國農業化學會誌 31(1):28-34。
- 邱德凱、靖大道、蕭樹東、曾民德與李繼強。1995。冬蟲夏草多糖脂質體對肝炎後肝硬化患者 T 細胞免疫調節作用的研究。中華消化雜誌 15(5):265-267。
- 徐泰浩。1999。冬蟲夏草與保健。中華傳統獸醫學會會刊 3(1):48-61。
- 食品分析方法手冊。1990。食品工業發展研究所。
- 陳傳盈、馮觀泉、許曉興、弗迪波、許少春與袁亞。1992。冬蟲夏草工業深層發酵研究。中草藥 23(8):409-416。
- 張家俊與陳文為。1992。天然冬蟲夏草及其培養菌絲體對能量代謝的影響。北京中醫學院學報 15(3):63-65。
- 張為憲、李敏雄、呂政義、張永和、陳昭雄、孫璐西、陳怡宏、張基郁、顏國欽、林志城與林慶文。1996。食品化學。華香園出版社。pp44。
- 蔣保季、孔祥環、王惠琴、尹學鈞、劉麗娟、馬忠杰、王虹、褚金花、沈家琴、張宏傳與肖中新。1995。發酵培育冬蟲夏草毒理研究 I、急性與亞慢性毒性研究。首都醫學院學報 16(3):198-203。
- 馮

頤、傅蓮瑛與袁淑蘭。1996。天然冬蟲夏草與蟲草菌提取液薄層對比試驗。天津藥學8(4):93-94。 17.郭育綺。1996。篩選具抑制活化腎間質細胞之冬蟲夏草子實體自然產物。中醫藥年報12(4):53-68。 18.郭育綺與陳建志。1999。冬蟲夏草中腫瘤細胞生長抑制因子之純化。產業科技發展學術合作論文集。pp321-328。 19.郭倩、周昌豔與高君輝。1998。無苦靈芝子實體多糖的研究。食用菌學報5(3):21-25。

20.程慶藥、于力方、師鎖柱與陳香美。1994。冬蟲夏草對5/6腎切除大鼠腎臟病理改變的影響。中華腎臟雜誌10(1):20。 21.解軍、郭欣、李培毅與徐衛東。1994。冬蟲夏草及人工菌絲體中蟲草菌素的定性定量研究。山西中醫10(4):36-38。 22.唐瑞菁與程梅萍。1992。靈芝培養基的探討 酵母抽出物的取代。國立雲林技術學院學報1:145-156。 23.Arcidiacono, S. and Kaplan, D. L. 1992. Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. Biotech. Bioeng. 39:281-286. 24.Chen, G. Z. and Chen, G. L. 1991. Effects of *Cordyceps sinensis* on murine T lymphocyte subsets. Chin. Med. J. 104(1):4-8. 25.Cheung, P. C. 1996. The hypocholesterolemic effect of extracellular polysaccharide from the submerged fermentation of mushroom. Nutr. Res. 11/12:1953-1957. 26.Chi, J. H., Oh, D. K., Kim, J. H. and Lebeault, J. M. 1991. Characteristics of novel high viscosity polysaccharide, methylan, produced by *Methylbacterium organophilum*. Biotech. Lett. 13(6):417-420. 27.Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal chem. 28(3):350-356. 28.De la Vega, M. G., Cejudo, F. J. and Panque, A. 1991. Production of exocellular polysaccharide by *Azotobacter chroococcum*. Appl. Biochem. Biotech. 30:273-284. 29.Gutierrez, A. and Prieto, A. and Martinez, A. T. 1996. Structural characterization of extracellular polysaccharides produced by fungi from the genus *Pleurotus*. Carb. Res. 281:143-154. 30.Gutierrez, A., Martinez, M. J., Almendros, G., Gonzalez-vila, F. J. and Martinez, A. T. 1995. Hyphal-sheath polysaccharides in fungal deterioration. Sci. Environ. 167:315-328. 31.Halpern, G. H. 1999. *Cordyceps chinensis*'s healing mushroom. pp1.

32.Hensel, A., Schmidgall, J. and Kreis, W. 1998. The plant cell wall- a potential source for pharmacologically active polysaccharides. Pharm. Acta Hel. 73:37-43. 33.Hosono, A., Lee, J., Ametani, A., Natsume, M., Hirayama, M., Adachi, T. and Kaminogawa, S. 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity form *Bifidobacterium adolescentis* M 101-4. Biosci. Biotech. Biochem. 64(2):312-316. 34.Isobe, Y., Endo, K. and Kawai, H. 1992. Properties of a highly viscous polysaccharide produced by *Bacillus* strain isolated from soil . Biosci. Biotech. Biochem. 56(4):636-639. 35.Israilides, C., Bocking, M., Smith, A. and Scanlon, B. 1994. A novel rapid coupled enzyme assay for the estimation of pullulan. Biotechnol. Appl. Biochem. 19:285-291. 36. Israilides, C. J., Smith, A., Harthill, J. E., Barnett, C., Bambalov, G. and Scanlon, B. 1998. Pullulan content of the ethanol precipitate from fermented agro-industrial wastes. Appl. Microbiol. Biotech. 49:613-617.

37.Kiho, T., Hui, J., Yamane, A. and Ukai, S. 1993. Polysaccharides in fungi XXXII. Hypoglycemic activity and chemical properties of polysaccharide from the cultural mycelium of *Cordyceps sinensis*. Biol. Pharm. Bull. 16(12):1291-1293. 38. Kiho, T., Hui, J., Yamane, A., Hui, J., Usui, S. and Ukai, S. 1996. Polysaccharides in fungi XXXVI. Hypoglycemic activity of a polysaccharides (CS-F30) from the cultural mycelium of *Cordyceps sinensis* and its effect on glucose metabolism in mouse liver. Biol. Pharm. Bull. 19(2):294-296. 39.Kitamura, S., Hori, T., Kurita, K., Takeo, K., Hara, C., Itoh, W., Tabata, K., Elgsaeter, A. and Stokke, B. T. 1994. An antitumor, branched (1-3)- β -D-glucan from a water extract of fruiting bodies of *Cryptoporus volvatus*. Carb. Res. 263:111-121. 40.Litchfield, J. H., Overbeck, R. C. and Davidson, R. S. 1963. Mushroom culture. Factors affecting the growth of morel mushroom mycelium in submerged culture. Agric. Food Chem. 11(2):158-162. 41.Lopez-barajas, M., Lopez-tamames, E. and Buxaderas, S. 1998. Improved size-exclusion high-performance liquid chromatographic method for the sample analysis of grape juice and wine polysaccharides . J. Chromato. A. 823:339-347. 42.Machova, E., Kvapilova, K., Kogan, G. and Sandula, J. 1999. Effect of ultrasonic treatment on the molecular weight of carboxymethylated chitin-glucan complex from *Aspergillus niger*. Ultra. Sonochem. 5:169-172.

43.Manzi, P. and Pizzoferrato, L. 2000. Beta-glucans in edible mushrooms. Food Chem. 68:315-318. 44.Muller, W. E. G., Weiler, B. E., Charubala, R., Pfleiderer, W., Leserman, L., Sobol, R. W., Suhadolnik, R. J. and Schroder, H. C. 1991. Cordycepin analogues of 2', 5' -oligoadenylate inhibit human immunodeficiency virus infection via inhibition of reverse transcriptase. Biochemistry 30:2027-2033. 45.Oh, S., Rheem, S., Sim, J., Kim, S. and Baek, Y. 1995. Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology. Appl. Environ. Microbiol. 61(11):3809-3814. 46.Pang, P. K. T., Shan, J. J. and Chiu, K. W. 1996. The cardiovascular effects of *Cordyceps sinensis* in normotensive rats. J. Chin. Med 7(2):153-167. 47.Peters, H., Herbst, H., Hessselink, P. M., Lunsdorf, H., Schumpe, A. and Deckwer, W. 1989. The influence of agitation rate on xanthan production by *Xanthomonas campestris*. Biotechnol. Bioeng. 34:1393-1397. 48.Roukas, T. and Liakopoulou-kyriakides M. 1999. Production of pullulan from beet molasses by *Aureobasidium pullulans* in a stirred tank fermentor . J. Food Eng. 40:89-94. 49.Smith, I. H. and Pace, G. W. 1982. Recovery of microbial polysaccharides. J. Chem. Technol. Biotechnol. 32:119-129. 50.Sone, Y., Okuda, R., Wada, N., Kishida, E. and Misaki, A. 1985. Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum* . Agric. Biol. Chem. 49(9):2641-2653. 51.Souza, M. C. O., Roberto, I. C. and Milagres, A. M. F. 1999. Solid-state fermentation for xylanase production by *Thermoascus aurantiacus* using response surface methodology. Appl. Microbiol. Biotechnol. 52:768-772. 52.Srinivasan, M., Natarajan, K. and Natarajan, G. 2000. Growth optimization of an ectomycorrhizal fungus with respect to pH and temperature in vitro, using design of experiments. Bioproc. Eng. 22:267-273. 53.Williams, D. L., Pretus, H. A. and Browder, I. W. 1992. Application of aqueous gel permeation chromatography with in-line multi-angle laser light scattering and differential viscometry detectors for the characterization of natural product carbohydrate pharmaceuticals. 1992. J. Liquid Chromatogr. 15:2297-2309. 54.Wood, P. J., Weisz, J. and Blackwell, B. A. 1991. Molecular characterization of cereal -D-glucans. Structural analysis of oat -D-glucans from different sources by high-performance liquid chromatography of oligosaccharides released by lichenase. 1991. Cereal Chem. 68(1):31-39. 55.Xu, H., Lee, S. H., S., Lee, S. F., White, R. L. and Blay J. 1999. Isolation and characterization of an anti-HSV polysaccharide from *Prunella vulgaris* . Antiviral Res. 44:43-54. 56.Yamaguchi, N., Yoshida, J., Ren, L. J., Chen,

H., Miyazawa, Y., Fujii, Y., Huang, Y. X., Takamura, S., Suzuki, S. and Zeng F. D.1990. Augmentation of various immune reactivities of tumor-bearing hosts with an extract of *Cordyceps sinensis* . *Biotherapy* 2:199-205. 57.Yang, F. C. and Liau, C. B. 1998. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* . *Proc. Biochem.* 33(5):547-553.