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Production of enzymes from *Lichtheimia ramosa* using Brazilian savannah fruit wastes as substrate on solid state bioprocessess

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Abstract

Background: Enzyme production by solid state bioprocess (SSB) using residues as substrate for microorganisms is an alternative for costs reduction and to avoid their disposal into environment. The aim of this work was to evaluate the physiology of the fungus *Lichtheimia ramosa* in terms of microbial growth and production of amylases, β-glucosidases, carboxymethylcellulase (CMCase), and xylanases, via SSB, utilizing wastes of the Brazilian savannah fruits bocaiuva (*Acrocomia aculeata*), guavira (*Campomanesia pubescens*) and pequi (*Caryocar brasiliense*) as substrate at different temperatures (25, 30, and 35ºC) during 168 hrs.
Results: Samples were taken every 24 hrs, which resulted in 8-points kinetic experiments to determine microbiological and enzymatic contents. The best substrate for β-glucosidase activity was pequi waste after 48 hrs at 30ºC (0.061 U/mL). For amylase activity, bocaiuva presented itself as the best substrate after 96 hrs at 30ºC (0.925 U/mL). CMCase activity was higher in guavira waste after 96 hrs at 35ºC (0.787 U/mL). However, the activity was more expressive for xylanase in substrate composed of bocaiuva residue after 144 hrs at 35ºC (1.802 U/mL).
Conclusions: It was concluded that best growth condition for *L. ramosa* is at 35ºC for all substrates and that xylanase is the enzyme with more potential in SSB, considering the studied Brazilian savannah fruit wastes.

Keywords: amylase, β-glucosidase, CMCase, *Lichtheimia ramosa*, xylanase.

INTRODUCTION

The concerns to reduce environmental pollution have encouraged the use of forestry, agro-industrial and urban waste sources through bioprocesses. The utilization of such materials as substrates in microbial cultivations to produce mushrooms, digestible cell proteins, organic acids, secondary metabolites, enzymes, fermentable sugars for the production of second-generation ethanol, among others, have been reported (Nigam and Singh 1996; Sánchez, 2009).

Regarding enzymes, the high price is still one of the major concerns for industrial application (Kang et al. 2004; Maciel et al. 2008). Enzymes are considered by their biotechnological importance. Their use has increased markedly in recent years (Graminha et al. 2008).

Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry, beyond its utilization in food, textile, chemical, pharmaceutical and detergent industries (Gupta et al. 2003; Demirkan et al. 2005; Sivaramakrishnan et al. 2006; Ravindar and Elangovan, 2013). The amylolytic enzymes are responsible for 25 to 33% of the world production of enzymes (Nguyen et al. 2002). The enzymes CMCase, xylanase, and β-glucosidase have potential to hydrolyze plant cell wall. CMCases and xylanases are applied in plant's digestion, juices and wine's clarification, aromas and pigments' extraction and pulp's bleaching (Kulkarni et al. 1999). β-glucosidases have received great attention due their applications in the release of flavour compounds in juices and wines (Gueguen et al. 1996; Martino et al. 2000; Baffi et al. 2011; González-Pombo et al. 2011), release of phenolic compounds with antioxidant activities from waste vegetables (Zheng and Shetty, 2000), synthesis of oligosaccharides and their use in processes for the enzymatic hydrolysis of cellulose (Daroit et al. 2008).

Vegetables wastes represent an alternative source for microbial growth aiming lower costs. Among the wastes commonly used for this purpose are sugar cane bagasse, corn cobs, corn and rice straws, rice and wheat bran, coffee husks, skins, peels and seeds of fruits (Sánchez, 2009; Singhania et al. 2009; Brijwani et al. 2010).

These residues are substrates that may permit the growth of microorganisms able to produce enzymes, especially fungi, which are considered the most suitable for solid state bioprocesses (SSB) because their hyphae can grow on the surface of solid particles and penetrate between them to colonize (Santos et al. 2004; Rajesh et al. 2010).

The Brazilian savannah has several fruit species traditionally used by the locals with great potential for agricultural use (Silva et al. 2008). The increased release of waste during the processing of these fruits has aroused the interest in its utilization by various segments of society (Lago-Vanzela et al. 2011).

Lichtheimia ramosa belongs to the Class Zygomycetes, which features rapid colonization. The Genus Lichtheimia (syn. *Mycocladus*, *Absidia*) is from the Order Mucorales, and includes saprotrophic microorganisms isolated from soil, and plant material in decomposition (Alastruey-Izquierdo et al. 2010). It has been reported as a producer of xylanase and CMCase and high producer of β-glucosidase in wheat bran (Gonçalves et al. 2013). Moreover, other microorganism from the same genus, *L. blakesleeana*, was recently described as phytase and xylanase producer (Neves et al. 2011).

Thus, the aim of this work was to evaluate the physiology of *L. ramosa* in terms of microbial growth and production of β-glucosidases, amylases, CMCases, and xylanases, via solid state bioprocesses, utilizing wastes of the Brazilian savannah fruits bocaiuva (*Acrocomia aculeata*), guavira (*Campomanesia pubescens*) and pequi (*Caryocar brasiliense*) as substrate at different temperatures (25, 30, and 35ºC).

MATERIALS AND METHODS

Microorganism

Lichtheimia ramosa was isolated from sugarcane bagasse, in Dourados, MS. It was maintained in inclined potato dextrose agar (PDA) (Himedia) (Gonçalves et al. 2013).

Substrates

Bocaiuva (*Acrocomia aculeata*), guavira (*Campomanesia pubescens*), and pequi (*Caryocar brasiliense*) fruit wastes were used as substrates. The fruits were collected from the proximities of Dourados, MS, Brazil. Bocaiuva waste, composed of fruit pericarp, mesocarp fiber (pulp-free) and endocarp were size reduced by knife mill (Bertel, Brazil) and selected by sieving through mesh sizes between 2.38 and 1.41 mm (Tyler mesh sizes 8 and 12). Residues of pequi were obtained by mechanical press (Hauber Macanuda, Brazil) to separate the peel from other parts of the fruit. Guavira waste, composed by peel and seeds, was extracted using depulper (Hauber Macanuda, Brazil). These were subsequently conditioned in plastic bags (Steriflex) and had moisture content (60%) and pH (5.5)

adjusted by distilled water and HCl (Vetec) addition, respectively. The thermal treatment was carried out by sterilization (121ºC, 15 min) before packing into the 500 mL Erlenmeyer flasks bioreactors (Silva et al. 2013).

Inoculum

Inoculum was prepared by transferring cells from an inclined PDA tube to PDA petri dishes. The plates were incubated in biological oxygen demand chamber (BOD) (Marconi, Brazil) at 25ºC for 7 days to serve as inoculum on the main cultivations. Inoculation of the fungus was carried out by the transfer of a 1 cm^2 square area of mycelium contained in a PDA petri dish to the Erlenmeyer flask, with the aid of a spatula previously sterilized (Silva et al. 2013).

Culture

The inoculated 500 mL Erlenmeyer flasks were maintained in three distinct BOD chamber at 25, 30 and 35ºC for 168 hrs. Every 24 hrs one flask was taken from each incubator, which resulted in 8-points kinetic experiments (including time zero) to determine microbiological and enzymatic contents.

Sampling for microbiological preparation

For each flask, 25 g of the homogenized medium was used for microbiological examination. During sampling and preparation a sterile environment was maintained. The sample was transferred to a same stomacher-bag and homogenized for 60 sec in a stomacher (Seward Stomacher® 400 Circulator, UK) with 225 g chilled saline peptone diluent (0.85% NaCl with 0.1% peptone) (Himedia). Further appropriate 10-fold dilutions of the homogenate were made with saline peptone diluent. For each dilution blank, two replicas were prepared.

Determination of *Lichtheimia ramose*

Lichtheimia ramosa was assayed according to classical methodology for fungal determinations: 0.1 mL from each appropriate dilution step was spread on the surface of PDA media into Petri dishes, which were incubated in BOD chamber at 25ºC for 5 days before colonies' count (ICMSF, 1978; ICMSF, 1986).

Aqueous enzymatic extraction

For the enzyme extraction, 50 mL of distilled water were added to Erlenmeyer flasks containing 5 g of cultivated mediums. The microorganisms were placed on an orbital shaker (Marconi, Brazil) (130 rpm, 35ºC, 30 min), paper filtered (Whatman, no. 1), and then centrifuged (ITR, Brazil) (3000 rpm, 5 min), yielding the crude enzyme extract for the determination of enzyme activity (adapted from Leite et al. 2007). The supernatant crude enzyme extract was used for determination of enzyme activities.

Amylase, CMCase and xylanase activities

The activities of xylanase, amylase and CMCase were determined on 0.1 mL of the enzyme filtrate, 0.9 mL of 0.2 M acetate buffer (pH 5.0) and 1% substrate (corn starch, carboxymethylcellulase and xylan, respectively) (Sigma). These activities were measured by the amount of reducing sugar present in the final enzymatic reaction, quantified by the 3,5-dinitrosalicylic acid method (DNS) (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme able of releasing 1 mmol of the respective product per minute of reaction.

β-glucosidase activity

β-glucosidase activity was determined with 50 µL of enzyme filtrate, 250 µL of acetate buffer 0.1 M, pH 5.0, and 250 µL of 4-nitrophenol β-glucopyranoside 4 mM (Sigma), which was allowed to react for 10 min at 50ºC. The enzymatic reaction was stopped with 2 mL of sodium carbonate 2 M (Impex), and the resulting product was quantified spectrophotometrically (Bioespectro, Brazil) at 410 nm (Leite et al.

2007). One unit of enzyme activity was defined as the amount of enzyme able to release 1 µmol of nitrophenol per minute of reaction.

RESULTS AND DISCUSSION

Microbial growth

L. ramosa presented a rapid colonization in all substrates at the different studied temperatures of 25, 30 and 35ºC (Figure 1). However, presented the best development at 35ºC in all substrates. Pequi waste showed itself the best substrate for microbial growth, reaching 6 log CFU/g (Figure 1c) while for bocaiuva and guavira wastes, reached maximums of 5 log CFU/g (Figures 1a and 1b, respectively). These results reveal the good capacity of *L. ramosa* for growing in different fruit residues.

However, the fact that for guavira waste the initial fungal counts (presumed *L. ramosa*) were around 1.5 fold lower than for the other substrates must be underlined. It was expected for all situations the same fungal counts for time zero, since that all cultivations were obtained from the same spawn.

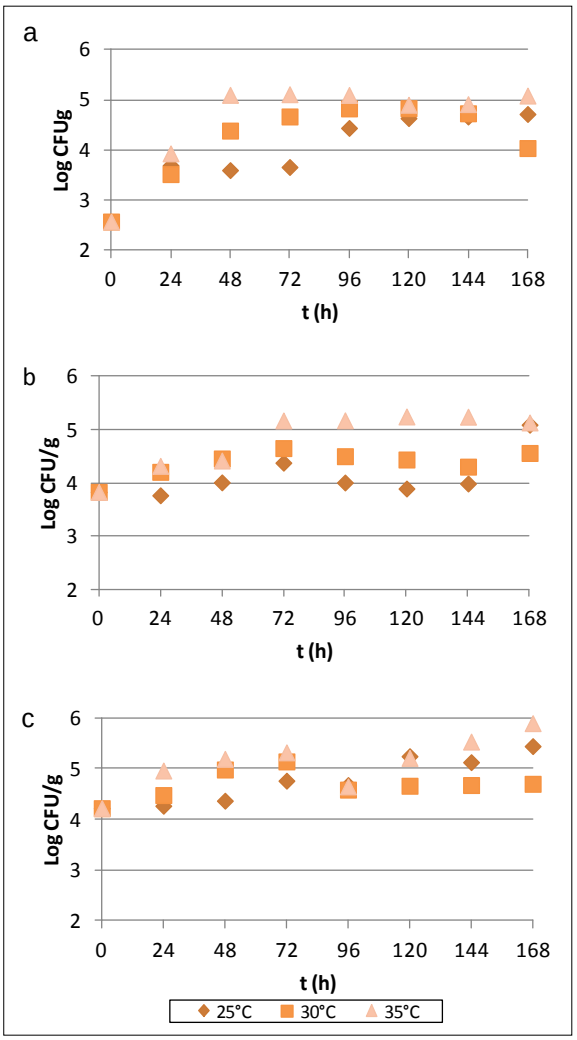

Fig. 1 Growth kinetics of *Lichtheimia ramosa* in fuits residues. (a) bocaiuva, (b) guavira and (c) pequi residues.

It is possible that the *L. ramosa* growth may have suffered some type of inhibition due to the presence of a specific compound in the bocaiuva waste substrate. This could occur because the time zero corresponds to the sampling after mixing the inoculum to the main cultivation medium. The time for homogenize the samples before preparing dilutions and plates might have also contributed to that. However, if it has occurred, the increase in cell counts in the first 24 hrs added to the own composition of the medium, which changes, may also have trigger out the rapid growth of *L. ramosa*.

In a previous study, *Pleurotus sajor-caju* grew in pequi and guavira waste substrates reached 5 and 5.5 log CFU/g after 5 days of cultivation, respectively, starting from an initial concentrations of 3.5 and 4 log CFU/g (Silva et al. 2013), being that the lower initial concentration was observed for guavira waste substrate.

Bacterial growth was not detected for none of the samples, ensuring that there was no contamination during the experiment and that the thermal treatment of substrates was enough to guarantee only the presence of the inoculated *L. ramosa*.

Enzyme activity analysis

Low enzyme activities of β-glucosidase, amylase, CMCase, and xylanase, and were observed in all substrates (Table 1). The best substrate for β-glucosidase activity was pequi waste after 48 hrs at 30ºC (0.061 U/mL) and 96 hrs at 35ºC (0.058 U/mL). For amylase activity, bocaiuva presented itself as the best substrate after 48 hrs at 25ºC (0.825 U/mL), 96 hrs at 30ºC (0.925 U/mL) and 72 hrs at 35ºC (0.812 U/mL). CMCase activity was higher in guavira waste after 120 hrs at 30ºC (0.656 U/mL) and 96 hrs at 35ºC (0.787 U/mL). However, the activity was more expressive after 144 hrs of cultivation for xylanase in substrates composed of bocaiuva (35ºC) and guavira (30ºC) residues, with 1.802 U/mL and 1.094 U/mL, respectively.

A higher β-glucosidase activity of 0.28 U/mL was reported with *L. ramosa* in sugar cane bagasse (Gonçalves et al. 2013). However, activities of 0.46 U/mL and 0.48 U/mL were also observed by this author for CMCase and xylanase, respectively, which are lower than those obtained here with fruit residues (Table 1). In the same study, 15.58 U/mL of β-glucosidase activity was detected in wheat bran cultivated with *L. ramosa* (Gonçalves et al. 2013).

Despite the difference between the enzyme activities in wheat bran and the other residues is related to the greater amount of macro and micro nutrients available in the wheat grain, compared to other agro-industrial wastes (Dias et al. 2003).

Amylase activities of 0.045 U/mL, 0.05 U/mL, 0.06 U/mL, and 0.08 U/mL were found in rice husk, banana peel, vegetable waste (potato, tomato, brinjal) and wheat bran, respectively, with the fungus *Aspergillus niger*. The authors classified these residues as potential good substrates for alpha amylases (Khan and Yadav, 2011). However, much higher amylase activities were observed for both pequi and guavira waste substrates in solid state bioprocesses with *P. sajor-caju* (Silva et al. 2013). For pequi waste substrate the authors found a higher production (9.4 U/g) after 20 days of cultivation. For guavira waste substrate, this enzyme activity remained stable (range of 6-8 U/g) during cultivation.

The reduced enzyme production obtained may be associated with the presence of free monosaccharides in the substrate composition. Generally, sugars readily assimilated by microorganism inhibit the synthesis of most microbial carbohydrases, considering the non-constitutive (inductive) nature of these enzymes. The results presented here confirm this hypothesis, since there was a low enzyme production, which was constant during cultivation, without production peaks during the evaluated period. As observed in previous works, that have reported lower enzyme production in crops using fresh fruit wastes.

CMCase activities in the range of 3 to 4 U/g were found on both pequi and guavira substrates with the fungos *P. sajor-caju* (Silva et al. 2013). The same authors found also no xylanase activity on pequi waste substrate, but showed 4.67 U/g activity in substrate composed with guavira residues. CMCase and xylanase activities of 4 U/mL and 8 U/mL, respectively were obtained with the fungus *Thermoascus aurantiacus* using fresh orange pulp production as substrate (Da-Silva et al. 2005). However, the production of 30 U/mL of CMCase and 64 U/mL of xylanase was observed using wheat

bran as substrate. According to the authors, the enzyme production by SSB can be influenced by the substrate characteristics, *e.g.* balance between carbon and nitrogen sources, solubilization rate of nutrients and microbial metabolites, content and composition of carbohydrates, presence of enzyme inducers or inhibitors and particles size.

Moreover, it was possible to observe a drop in enzymatic activity at the end of cultivation, coinciding with a long stabilization period of microbial growth in all the substrates (Figure 1). This reduction of enzyme activity can be explained by changes in the growth medium due to metabolic activity of the microorganism. Some parameters such as pH, incubation time, moisture, oxidizing agents, and even proteases production may inactivate extracellular enzymes previously secreted, as observed in different studies using plant wastes as solid state substrates (Dyk et al. 2010; Pal and Khanum 2010; Pribowo et al. 2012; Silva et al. 2013).

Despite the lower productivity of enzymes with residues, these substrates must be taken into consideration because they have low cost (Damaso et al. 2000). Therefore, the study of agro-industrial residues in bioprocesses may provide alternative substrates and also helps to solve environmental problems, which are otherwise caused by their disposal (Reddy et al. 2003).

We believe in the biotechnological potential of isolated strain, especially if considered that clinical cases with the fungus *L. ramosa* are mostly associated with immunosuppressed patients (Bibashi et al. 2013). The description of new microbial strains, producers of enzymes of industrial interest, should be highlighted even for potentially pathogenic strains, considering the advances of genetic engineering techniques, which allow the extraction and insertion of genetic material in different cellular structures.

CONCLUDING REMARKS

L. ramosa grew in Brazilian savannah fruit wastes through solid state bioprocesses showed good adaptation to the substrates bocaiuva, guavira and pequi at 25, 30 and 35ºC. However the best development of *L. ramosa* was obtained at 35ºC. The highest activities were observed for xylanase after 144 hrs cultivation in bocaiuva (35ºC) and guavira (30ºC) residues.

In the face of the low enzyme activities observed, *L. ramosa* has potential for enzyme production at optimized conditions. Moreover, it is important to highlight that the availability of these residues justifies any effort to find biotechnological applications to them.

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Table 1. Enzymatic activity of β-glucosidase, amylase, CMCase and xylanase produced on substrates with bocaiuva, guavira and pequi residues with *Lichtheimia ramosa* cultivated at 25ºC, 30ºC and 35ºC.

Substrate	Time (hrs)	β-glucosidase (U/ml)			Amylase (U/mL)			CMCase (U/ml)			Xylanase (U/ml)		
		25ºC	30ºC	35ºC	25ºC	30ºC	35ºC	25ºC	30ºC	35ºC	25ºC	30ºC	35ºC
Bocaiuva	0	0.008±0.001	0.008±0.001	0.008±0.001	0.445±0.05	0.445±0.05	0.445±0.05	0.294±0.03	0.294±0.03	0.294±0.03	0.412±0.02	0.412±0.02	0.412±0.02
	24	0.020±0.003	0.009±0.004	0.010±0.002	0.254±0.03	0.337±0.09	0.261±0.01	0.359±0.01	0.333±0.01	0.300±0.01	0.825±0.09	0.468±0.01	0.637±0.06
	48	0.011±0.001	0.010±0.001	0.008±0.001	0.363±0.01	0.350±0.01	0.248±0.05	0.385±0.03	0.333±0.05	0.352±0.02	0.456±0.03	0.412±0.02	0.556±0.01
	72	0.011±0.002	0.010±0.003	0.011±0.001	0.254±0.03	0.350±0.03	0.261±0.01	0.300±0.05	0.333±0.03	0.313±0.01	0.412±0.02	0.412±0.05	0.812±0.05
	96	0.009±0.005	0.008±0.004	0.010±0.005	0.273±0.01	0.356±0.06	0.420±0.10	0.300±0.01	0.294±0.02	0.307±0.04	0.475±0.03	0.925±0.04	0.537±0.09
	120	0.013±0.008	0.008±0.001	0.010±0.003	0.242±0.05	0.242±0.01	0.541±0.02	0.581±0.06	0.346±0.04	0.287±0.02	0.668±0.05	0.706±0.09	0.431±0.07
	144	0.015±0.002	0.008±0.001	0.008±0.002	0.242±0.03	0.312±0.07	1.802±0.07	0.326±0.02	0.313±0.05	0.294±0.04	0.431±0.06	0.768±0.07	0.493±0.02
	168	0.008±0.004	0.014±0.003	0.022±0.004	0.369±0.07	0.305±0.05	0.484±0.12	0.339±0.01	0.379±0.02	0.313±0.05	0.643±0.07	0.706±0.04	0.475±0.04
Guavira	0	0.008±0.001	0.008±0.001	0.008±0.001	0.343±0.11	0.343±0.11	0.340±0.11	0.412±0.01	0.412±0.01	0.412±0.01	0.412±0.04	0.412±0.04	0.412±0.04
	24	0.024±0.002	0.036±0.004	0.032±0.004	0.615±0.05	0.343±0.02	0.449±0.05	0.493±0.06	0.406±0.03	0.675±0.00	0.587±0.07	0.412±0.03	0.412±0.01
	48	0.026±0.003	0.023±0.003	0.024±0.003	0.461±0.05	0.408±0.06	0.343±0.05	0.475±0.03	0.518±0.07	0.468±0.02	0.543±0.02	0.456±0.06	0.412±0.02
	72	0.018±0.002	0.020±0.001	0.050±0.002	0.502±0.04	0.698±0.02	0.455±0.02	0.406±0.01	0.406±0.06	0.481±0.06	0.456±0.03	0.450±0.02	0.481±0.01
	96	0.033±0.005	0.015±0.001	0.013±0.001	0.396±0.04	0.656±0.05	0.437±0.06	0.512±0.02	0.425±0.02	0.787±0.09	0.437±0.01	0.425±0.07	0.418±0.03
	120	0.022±0.001	0.028±0.002	0.015±0.001	0.402±0.01	0.408±0.01	0.455±0.01	0.418±0.07	0.656±0.09	0.637±0.12	0.431±0.03	0.543±0.01	0.406±0.08
	144	0.023±0.001	0.013±0.001	0.031±0.004	0.473±0.07	1.094±0.07	0.337±0.02	0.412±0.08	0.543±0.07	0.550±0.04	0.543±0.05	0.631±0.09	0.418±0.06
	168	0.014±0.002	0.027±0.003	0.018±0.001	0.408±0.01	0.461±0.05	0.343±0.01	0.500±0.02	0.418±0.01	0.412±0.05	0.556±0.01	0.406±0.03	0.406±0.01
Pequi	0	0.008±0.001	0.008±0.001	0.008±0.001	0.343±0.11	0.343±0.11	0.343±0.11	0.406±0.04	0.406±0.04	0.406±0.04	0.406±0.02	0.406±0.02	0.406±0.02
	24	0.031±0.000	0.010±0.001	0.014±0.004	0.751±0.04	0.360±0.04	0.431±0.04	0.643±0.09	0.406±0.01	0.462±0.07	0.431±0.01	0.506±0.01	0.681±0.09
	48	0.018±0.001	0.061±0.003	0.010±0.001	0.609±0.02	0.621±0.06	0.473±0.05	0.431±0.03	0.506±0.02	0.456±0.05	0.493±0.03	0.443±0.04	0.456±0.01
	72	0.009±0.001	0.020±0.001	0.026±0.005	0.609±0.08	0.609±0.08	0.585±0.06	0.418±0.01	0.431±0.04	0.406±0.03	0.456±0.02	0.406±0.05	0.512±0.04
	96	0.036±0.004	0.035±0.004	0.058±0.009	0.579±0.09	0.497±0.06	0.514±0.02	0.406±0.01	0.443±0.04	0.406±0.01	0.475±0.06	0.537±0.08	0.512±0.07
	120	0.029±0.003	0.011±0.005	0.027±0.004	0.609±0.01	0.449±0.02	0.414±0.01	0.406±0.08	0.406±0.01	0.475±0.03	0.412±0.02	0.543±0.06	0.481±0.02
	144	0.009±0.000	0.011±0.003	0.030±0.007	0.556±0.03	0.384±0.01	0.520±0.03	0.481±0.09	0.487±0.05	0.475±0.07	0.406±0.04	0.412±0.01	0.681±0.08
	168	0.036±0.005	0.033±0.006	0.029±0.003	0.650±0.08	0.508±0.03	0.538±0.09	0.425±0.06	0.406±0.06	0.406±0.04	0.406±0.03	0.518±0.09	0.500±0.04

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