Food Research International 89 (2016) 764–772

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Aseptic artificial fermentation of cocoa beans can be fashioned to replicate the peptide profile of commercial cocoa bean fermentations

Warren A. John [a], Neha Kumari [a], Nina L. Böttcher [a], Kouame Jean Koffi [b], Sergio Grimbs [a], Gino Vrancken [b], Roy N. D'Souza [a], Nikolai Kuhnert [a], Matthias S. Ullrich [a,*]

[a] Department of Life Sciences and Chemistry, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany
[b] Barry Callebaut Belgium N.V., Aalstersestraat 122, 9280 Lebbeke-Wieze, Belgium

ARTICLE INFO

Article history:
Received 17 August 2016
Received in revised form 10 October 2016
Accepted 10 October 2016
Available online 12 October 2016

Keywords:
Artificial fermentation
Flavour precursors
Cocoa
Peptides
Proteases

ABSTRACT

The fermentation of cocoa beans is essential for the generation of flavour precursors that are required later on to form the flavour components of chocolate. From the many different precursors that are generated, oligopeptides and free amino acids comprise a significant proportion as some of them form Maillard reaction products during the roasting process. Therefore, the diversity of peptides is an important contributing factor to the quality of a fermentation which is in turn controlled by proteolytic activity within the cocoa bean, and is driven by changes in the presence of fermentation by-products as a result of microbial activity outside the bean. Being able to control proteolytic activity within the bean using only the presence of fermentation by-products would prove a valuable tool in the study of these proteases and the processing of cocoa storage proteins. Thus, this tool would help elucidate key mechanisms that generate the components responsible for flavour. In this study, we describe an artificial fermentation system, free from microbial activity, which is able to replicate proteolytic degradation of protein as well as to generate similar peptide fragments as seen during a commercial fermentation. It was also found that acidification is a main contributor to protein degradation.

1. Introduction

Farming of the cocoa tree (*Theobroma cacao*) has been carried out for over 3500 years, having its roots in Mesoamerican societies and now having spread to all tropical regions of the world with Ivory Coast and Ghana contributing the most to cocoa cultivation (Dreiss & Greenhill, 2008; Ismail et al., 2009). Intense cocoa farming has resulted in vast numbers of cocoa hybrids designed for resistance to unfavourable weather conditions and pathogenicity as well as for unique chocolate flavour profiles (Motamayor, Risterucci, Heath, & Lanaud, 2003; Wood & Lass, 2001) such that each farm or institution can have its unique cocoa to sell on the market.

The chocolate production process involves many stages that are crucial for the development of a pleasant flavour profile, among which are selection of the hybrid, bean fermentation and bean roasting (Afoakwa, Paterson, Fowler, & Ryan, 2008). Fermentation is a key process as it is involved in the development of flavour precursors from the cocoa bean. Flavour precursors and flavour components of chocolate can be diverse in their nature, ranging from sugars, peptides and amino acids to complex products such as Maillard reaction products, theobromine, diketopiperzines, etc. (Afoakwa et al., 2008; Camu et al., 2008; Stark & Hofmann, 2005). Over the course of the fermentation, a repertoire of yeasts, lactic acid bacteria, and acetic acid bacteria on the exterior of the bean commandeer the process in respective succession (Pereira, Miguel, Ramos, & Schwan, 2012). The pulp, being rich in nutrients and low in pH, in combination with decreasing oxygen concentration, allow yeasts to thrive. The yeasts degrade the pulp with pectinolytic enzymes, metabolizing sugar and citric acid and as a result, produce ethanol and raise pH (Papalexandratou & De Vuyst, 2011; Rombouts, 1953; Schwan & Wheals, 2004). Yeasts contribute to flavour by the generation of volatile substances in the first stages of the fermentation (Schwan & Wheals, 2004; Steensels & Verstrepen, 2014). Anaerobic conditions and high CO_2 concentrations favour the growth of lactic acid bacteria, albeit for a brief period. After the beans are turned (typically on the second day of fermentation), oxygen is reintroduced and populations of acetic acid bacteria take over, producing acetic acid from the remaining ethanol in an exothermic reaction thereby raising the bean pile's temperature (Camu et al., 2007). These fermentation products then diffuse into the bean over the course of the fermentation, causing the death of the embryo and a cascade of various enzymatic reactions and structural changes inside the cotyledons (Biehl, Passern, & Sagemann, 1982; Lefeber, Gobert, Vrancken, Camu, & De Vuyst, 2011).

The various fermentation-driven changes within the cocoa bean also involve the degradation of proteins (up to 20% of bean mass), the majority of which consists of 52% albumin (a 21-kDa storage protein) and 43%

* Corresponding author.
E-mail address: m.ullrich@jacobs-university.de (M.S. Ullrich).

http://dx.doi.org/10.1016/j.foodres.2016.10.011
0963-9969/

vicilin-like globular storage protein, here on referred to simply as vicilin (Voigt & Biehl, 1993). The vicilin protein is preferred over albumin for breakdown by the native activities of a speculated aspartic endoprotease, aminopeptidase and a carboxypeptidase (Hansen, Del Olmo, & Burri, 1998; Laloi, Mccarthy, Morandi, Gysler, & Bucheli, 2002; Lerceteau, Rogers, Pétiard, & Crouzillat, 1999; Voigt, Heinrichs, Voigt, & Biehl, 1994). The activities of those enzymes are stimulated during the conditions generated during fermentation. It has been understood from the fermentation-like incubations carried out by Biehl et al. that as acetic acid diffuses into the bean during fermentation, there is a profound impact on subcellular structure within the cotyledons, affecting protein and lipid bodies and thus, also impacting proteolysis within the cotyledons (Biehl & Passern, 1982; Biehl et al., 1982). The endoprotease that is active in protein cleavage into peptides is known to have a pH optimum of 3.0, spurred on by the diffusion of acetic acid into the bean (Amin, Jinap, Jamilah, Harikrisna, & Biehl, 2002). These peptides and free amino acids can go on to react with sugars and polyphenols in Maillard reactions in the roasting process (Bonvehí & Coll, 2002; Mohr, Landschreiber, & Severin, 1976; Jürgen Voigt, Janek, Textoris-Taube, Niewienda, & Wöstemeyer, 2016; Ziegleder, 1991), thereby making them key precursors required for chocolate flavour.

The aim of this study was to model cocoa fermentation in such a way that internal processes in the bean would be triggered solely by the presence of fermentation by-products in a succession of buffer incubations, without any microbial contribution. Changes in the environmental concentrations of these fermentation products were tracked and degradation products of proteins were then analysed with protein quantification assays and high-resolution HPLC-MS. The results were compared to those of a commercial spontaneous fermentation.

2. Material and methods

2.1. Chemicals and reagents

Tris-HCl (Pufferan®, ≥99.5%), yeast extract (bacteriology grade), sodium dodecyl sulphate (SDS, ≥99.5% electrophoresis grade), HPLC-grade water (Rotisolv®), glycerol (86%), acrylamide (Rotiphorese® Gel 30: 37,5:1), acetonitrile (ACN, Rotisolv® HPLC ultra gradient grade), citric acid (≥99.5%), ethanol (≥96%, denatured with 1% MEK), lactic acid (90% synthetic grade), fructose (≥99.5% for Biochemistry) and D-glucose monohydrate (≥99.5%) were purchased from Carl Roth (Karlsruhe, Germany). Dithiothreitol (DTT, Biochemica), acetone (100%, Biochemica), TEMED, ammonium persulphate (analytical grade), sulphuric acid (90–91%, pure), acetic acid (100%, analytical grade) and methanol (100%, analytical grade) were purchased from Applichem (Darmstadt, Germany). Isopropanol (100%, analytical grade) and bromophenol blue sodium salt (research grade) were purchased from Serva Electrophoresis (Heidelberg, Germany). Formic acid (≥98% for mass spectrometry) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). 2D Quant Kit was purchased from GE Healthcare Biosciences (New Jersey, USA). Coomassie® Brilliant blue G-250 (electrophoresis grade) was purchased from Merck (Darmstadt, Germany).

2.2. Artificial fermentation

All equipment used was either wiped with 70% ethanol or autoclaved in order to exclude any microbial contribution to the system. Cocoa beans of the hybrid C308XC5 from Ivory Coast were removed from the pods upon shipment and immediately de-pulped under high pressure water. Beans were rinsed in 70% ethanol for 1 min to get rid of any remaining microbes on the surfaces of the beans. 100 g portions of beans were placed inside 500-mL bottles along with 90 mL buffer and the headspace was flushed with either nitrogen or compressed air through a 0.2-μm filter depending on the day of incubation. The bottles were placed in incubators and the buffers exchanged every 24 h under sterile conditions inside a laminar flow work bench. Two trials of artificial fermentation were run along with a control involving low concentrations of the buffer constituents. Table 1 shows an overview of the various trials conducted. Trial 1 was carried out to mimic the changes of fermentation by-products as experienced in a commercial fermentation. Trial 2 was conducted to investigate the effect of pH. Trial 2 had two control incubations. One control was maintained in Buffer C1 at pH 3 instead of pH 2 for the whole length of the fermentation without buffer exchange. The other control was maintained in Buffer C5 at pH 5 instead of pH 6 in the same manner. Sampling was done in triplicate after every 24 h where three bottles representing three biological replicates were removed and the beans and buffers were collected and stored at −20 °C. Shells of the beans were removed and the beans were ground to powder and stored at −20 °C. The remaining bottles had their buffers exchanged, were flushed once again and placed at the respective incubation temperature. Sterility of the system was confirmed by plating on lysogeny broth (LB) medium, acetic acid medium, glucose-yeast extract-carbonate (GYC medium) and malt extract agar to check specifically for the growth of yeasts, lactic acid bacteria, and acetic acid bacteria as well as any other microorganisms that could potentially thrive in these buffers.

2.3. Commercial fermentation

Pods of the hybrid C308XC5 from Ivory Coast were harvested in December 2013 in the estate of Champ Semencier de Mafere where the average age of the trees was 30 years. The beans were removed from the pods within 12 h of harvesting with the placenta, broken beans, and the pod husks being discarded. Beans were placed into a pile of 100 kg, and fermentation was carried out for a period of seven days. Beans in the pile were turned after 48 and 96 h. Sampling was done from the middle of the pile in portions of 2 kg and were immediately stored at −20 °C and shipped to Jacobs University Bremen, Germany on dry ice within three days. Shells of the beans were removed and the beans were ground to powder and stored at −20 °C.

2.4. Protein extraction

1 mL of protein extraction buffer, containing 100 mM Tris-HCl, 1% SDS and 1% DTT, was added to 250 mg of cocoa bean powder in a 2-mL centrifuge tube, vortexed briefly, and kept on a gently turning head-over-head rotator at 4 °C for 1 h. The mix was vortexed again and centrifuged at 16,000*g* for 20 min at 4 °C. The resulting aqueous fraction was carefully pipetted out into a pre-equilibrated Vivaspin 2 column (Sartorius, Goettingen, Germany) containing a hydrosart

Table 1
Buffers and their components present in incubations of artificial fermentation along with temperature conditions.

Fermentation	Buffer	Citric acid [g/L]	Ethanol [g/L]	Acetic acid [g/L]	Lactic acid [g/L]	Temperature [°C]	pH
Trial 1	A1	15	80	0	0	37	2.3
	A2	10	80	15	30	45	2.0
	A3	10	40	15	30	30	2.0
Control with low concentrations	B1	1.2	0.79	0.17	0.31	37	2.7
	B2	0.9	1.18	0.40	1.2	45	2.6
	B3	0.1	1.58	0.53	1.7	30	2.7
	B4	0.1	0.79	0.53	1.7	30	2.7
	B5	0.1	0.40	0.53	1.7	30	2.7
Trial 2	C1	15	22.5	15	0	35	2.0[a]
	C2	10	22.5	15	0	35	3.0[a]
	C3	5	45	10	0	35	4.0[a]
	C4	0	45	0	0	35	5.0[a]
	C5	0	90	0	0	35	6.0[a]

Beans were incubated in a succession of buffers at a constant temperature for 24-hour periods for each fermentation trial.
[a] pH values that were controlled for before incubation.