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Rothia aeria gluten degradation under gastro-duodenal conditions

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THESIS

ROTHIA AERIA **GLUTEN DEGRADATION UNDER GASTRO-DUODENAL CONDITIONS**

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DEDICATION

To my adoring parents,

Chin-Cheng Yang & Ching-Jung Shih

To my beloved brother,

Chin-Yun Yang

For their love, encouragement, and support enthusiasm.

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ROTHIA AERIA GLUTEN DEGRADATION UNDER GASTRO-DUODENAL CONDITIONS

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ABSTRACT

Introduction: Celiac disease is a T-cell mediated-inflammatory disorder of the small intestine precipitated by gluten ingestion. Gluten can be effectively degraded by *Rothia aeria*, a natural resident oral microbe. *Rothia* bacteria can potentially be developed as a first probiotic for celiac disease.

Aims: To select the optimal culture conditions for *R. aeria* enzyme expression *in vitro* and determine the ratio of *R. aeria* to gluten to achieve digestion within 2 hr incubation.

Materials and Methods: *R. aeria* were cultivated in Brain Heart Infusion (BHI) with different strength (4%, 20% and 100%), and chemical defined media M9 supplemented with different carbon sources (glucose, succinate, glycerol, or casein). The effect of incubation time and temperature (28°C and 37°C) on the enzyme expression of the bacteria grown in BHI was also assessed. Gliadin degradation was investigated by incubating a fixed gliadin concentration (250 µg/ml) with different amount of *R. aeria* cell (OD_{620} 1.0, 0.5, 0.25), and analyzed by SDS-PAGE.

Results: The OD_{620} of 24 hr *R. aeria* culture in 4%, 20%, and 100% BHI were 0.240, 0.932, and 2.033, respectively. No bacterial growth was observed in M9 broth +2% glucose, succinate, or glycerol, but grew well in M9 broth +2% casein with OD_{620} 19.54. *R. aeria* exhibited

highest activity when grown in 100% BHI (0.22 AU/min) and lowest in 4% BHI (0.1 AU/min). Enzyme activities in M9+2% casein were low (0.035 AU/min), not in proportional to the OD of *R. aeria* culture. The specific enzyme activities of *R. aeria* in 100% BHI was high in log phase (9 to 12 hr or 12 to 18 hr), but the yield of total activity was less than that of in stationary phase (20 to 44 hr). The activity of the cells grown at 37°C was higher than at 28°C. *R. aeria* suspension with an OD_{620} of 1.0 exhibited rapid degradation of gliadins at 250 ug/ml.

Conclusions: Full strength BHI broth and 20 to 44 hr cultivation at 37°C are considered as optimal cultivation condition to obtain a *R. aeria* cell culture with high enzyme activity. Starvation condition do not enhance enzyme expression.

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LIST OF ABBREVIATIONS

BHI .... Brain Heart Infusion

CD .... Celiac Disease

ELISA .... Enzyme Linked Immunosorbent Assay

EP-B2 .... Endoprotease B2

HLA-DQ .... Human Leukocyte Antigen DQ isoform

Hr .... Hour

KDa .... kilo Dalto

Min .... Minute

MW .... Molecular Weight

OD .... Optical Density

PEP .... Prolyl endopeptidases

R. aeria .... *Rothia aeria*

Suc-AAPF-pNA .... Succinyl Ala Ala Pro Phe p-nitroanilide

1. INTRODUCTION

1.1. CELIAC DISEASE

1.1.1. Definition

Celiac disease (CD) is an inherent and widespread autoimmune disorder of the upper small intestine (Green and Cellier, 2007) It is triggered by ingested gluten proteins found in wheat, rye, and barley in genetically predisposed individuals (Green and Jabri, 2003; Kagnoff, 2005; Sollid, 2000).

1.1.2. Etiology

CD is caused by the ingestion of gluten, which are present in wheat, barley, and rye. Gluten comprises gliadins and glutenins. Gliadin is the alcohol soluble fraction of gluten and contains most of the toxic components. The gluten proteins are proline- and glutamine-rich and are difficult to be digested by mammalian digestive enzymes. Undigested gliadin fragments remain after incubation with gastric, pancreatic, and intestinal brush-border membrane proteases acting in the stomach and the small intestine (Shan et al., 2002).

In patients with CD, the immune response to gliadin fractions lead to an inflammatory reaction, mainly in the upper small intestine, characterized by the infiltration of the epithelium and the lamina propria with inflammatory cells. This response is attributed to the innate and the adaptive immune systems in the epithelium and in the lamina propria, respectively (Green and Cellier, 2007). In the epithelium, gliadin damages the epithelial cell resulting in increased expression of interleukin-15 by enterocytes which activates intraepithelial lymphocytes in turn. These lymphocytes become cytotoxic and kill enterocytes.

After entering of gliadins to the lamina propria, they are deaminated by tissue transglutaminase (TG2), leading an increase in their immunogenicity by facilitating a strong interaction with HLA class II genes HLA-DQ2 or HLA-DQ8 on the surface of antigen-presenting cells such as macrophages, dendritic cells, and B cells (Henderson et al., 2007; Kim et al., 2004; Molberg et al., 1998). The adaptive response is mediated by gliadin-reactive CD4+ T-helper 1 cells in the lamina propria that recognize these negatively charged gliadin peptides presented in the HLA context. The T cells produce cytokines (Sollid, 2002), particularly interferon-γ (Nilsen EM, 1998), which ultimately results in tissue remodeling and villous atrophy, and crypt hyperplasia (Kagnoff, 2005; Mohamed et al., 2006).

The clinical symptoms of CD vary greatly from different ages. Infants and young children generally have diarrhea, abdominal distention, and failure to thrive. Furthermore, vomiting, irritability, anorexia, and constipation are also common. The most seen in adults is diarrhea, which may be accompanied by abdominal pain. Extraintestinal symptoms such as short figure, neurologic symptoms, or anemia are often presented in older children and adults (D'Amico et al., 2005). Less common presentations include constipation, weight loss, dermatitis herpetiformis, hypoproteinemia, hypocalcemia, and elevated liver enzyme levels (Green, 2005).

A 33-mer peptide from α2-gliadin contains 6 partly overlapping HLA-DQ2-binding amino acid sequences that can be deaminated by TG2. This peptide is considered a celiac “superantigen” (Shan et al., 2002). The mechanism of the interaction between the processes in the epithelium and in the lamina propria has not been fully elucidated yet.

The presence of HLA-DQ2 or HLA-DQ8 proteins, products of two of the HLA genes, on antigen presenting cells is necessary for the induction of CD (Sollid and Lie, 2005). However, their presence is not sufficient for the development of the disease. Studies in siblings and identical twins suggested that the contribution of HLA genes to the genetic component of CD is less than 50% (Greco et al., 2002). This implicates other factors, e.g. genetic and/or environmental factors as contributors to CD. Some non-HLA genes which may influence susceptibility to the disease have been identified, specially CTLA4, Myosin IXB, KIAA1109, TENR, IL2, IL21, RGS1, IL18RAP, IL18R1, CCR1, CCR2, CCRL2, CCR3, CCR5, XCR1, IL12A, LPP, TAGAP, and SH2B3 (Adamovic et al., 2008; Babron et al., 2003; Dema et al., 2009; Djilali-Saiah et al., 1998; Garner et al., 2009; Greco et al., 1998; Haimila et al., 2009; Koskinen et al., 2009; Liu et al., 2002; McIntire et al., 2008; Monsuur et al., 2005; Naluai et al., 2000; Naluai et al., 2001; Romanos et al., 2009; van Belzen et al., 2004; van Heel et al., 2007; Woolley et al., 2002). Family history is also a factor to those who have CD (Fasano et al., 2003), also Down syndrome (Shamaly et al., 2007), Turner's syndrome (Bettendorf et al., 2006), or type I diabetes (Goh and Banerjee, 2007) are associated to them. Environmentally, early exposure of infants to dietary gluten (Norris et al., 2005), early infection with enteropathic viruses (Stene et al., 2006); (Pavone et al., 2007), or a change of the bacterial flora (Collado et al., 2007; Collado et al., 2008; 2009; Zanoni et al., 2006) have been identified as contributing factors to CD development in childhood.

1.1.3 Diagnosis of Celiac Disease

CD is recognized as a relatively common condition that may be diagnosed at any age. The diagnosis of it requires both a duodenal biopsy and a positive response to a gluten free diet. The biopsy taken by gastroduodenoscopy can detect signs of intraepithelial lymphocytosis, infiltration of the lamina propria with T cells and plasma cells, crypt hyperplasia, and villous atrophy (Rostom et al., 2006). At least four biopsies should be taken to prove CD because the celiac lesions in small intestine could be patchy. Also the serological test is important to detect CD. Obviously the IgG and IgA antibodies will disappear from the result of serological test once the gluten free diet is applied.

The histologic grading in CD suggested by Marsh is widely used now. It is classified in to five degrees as 0, I, II, III, IV, from normal mucosal architecture to hypoplastic condition. It has been suggested to consider clinical signs and serology accompanied with biopsies to detect the CD more correctly[EH1] (Marsh, 1992).

1.1.4 Current treatment and therapeutic avenues

The therapy for CD is a lifelong strict adherence to a gluten-free diet; however, the response to therapy is poor in up to 30% of patients (O'Mahony et al., 1996). Thus alternative treatments are desired (Sollid and Khosla, 2005; (Di Sabatino and Corazza, 2009). The major objective of alternative treatment would be to facilitate the safe ingestion of low amounts of gluten without any intestinal inflammation. Several current therapeutic avenues effective in different body locations including a) intraluminal therapies, b) transepithelial uptake, c) frustrating the adaptive immune response, d) immune modulation and e) induction of tolerance to gluten.[EH2]

[YC3]

a) Intraluminal therapies

The purpose of intraluminal therapies is either to reduce gluten immunogenicity or to prevent gluten from contacting intestinal epithelium.

1) Wheat variants: Some wheat strains with lower immunogenicity can be selected from existing varieties or can be created by genetic modification. The bread wheat (hexaploid *Triticum aestivum*) is the most widely consumed wheat variety. It is generated by hybridation between durum wheat (tetraploid *Triticum turgidum*) and the diploid *Triticum tauschii* (Ozkan H, 2001). In the duodenal biopsies of CD patients, tetraploid wheat-derived gluten peptides showed lower toxicity than the hexaploid wheat gluten fraction. Based on this it can be concluded that durum wheat, although not harmless, could be much better tolerated than bread wheat in CD (Auricchio et al., 1982).

2) Genetic modification: The effect of genetic deletion of certain gliadin genes has been studied in deletion variants of *T. aestivum,* lacking one locus containing the gluten genes, by using RNA interference methodology. Complete deletion of the α-gliadin locus on chromosome 6 caused a decrease in total T-cell stimulatory epitopes also impaired the baking properties whereas deletion of γ-gliadins, ω-gliadins, and low molecular weight glutenins on chromosome 1 lowered the immune stimulatory capacity without compromising baking properties (Schuppan et al., 2009). The genetic modification can also be accomplished by mutagenesis, transgenesis, and genome editing. The treatment of wheat with radiation or chemicals leading to mutation is an effective way to generate novel genetic diversity and has been used to produce over 300 crop cultivars (http://mvgs.iaea.org/Defult.aspx). The identification of the gene sequence level has been checked by PCR-based screening, known as TILLING (Targeting Induced Local Lesions in Genomes) (McCallum et al., 2000). Mutagenesis and TILLING platforms are widely available for wheat now (Slade et al., 2005); (Chen et al., 2012); (Sparla et al., 2014). On the other hand, transgenesis by either biolistic or Agrobacterium-mediated systems (Sparks and Jones, 2009; Wu et al., 2009) can down regulate endogenous genes or adding novel genes. Moreover, genome editing is a new technology in which highly specific changes are made to genomes without leaving any foreign DNA.

3) Pretreatment of flours: Another approach to eliminate gluten from the dough is by fermentation with certain *lactobacilli* ("sourdough" *lactobacilli*) that can digest the proline/glutamine-rich gluten peptides and thereby reduce immunotoxicity (De Angelis et al., 2006; Di Cagno et al., 2002; Rizzello et al., 2007).

Yet another approach is to treat the whole wheat flour with a low-molecular-weight microbial TG derived from *Streptomyces moboraensis* to crosslink glutamines residues thereby preventing their deamination in the lamina propria (Yokoyama et al., 2004).

4) Neutralizing gluten antibodies[EH4][YC5]: Because orally ingested IgG is highly resistant to gastric acidity and remains about 50% active in neutralizing capacity when reaching the small intestine and is easy and cheap to produce, this gluten-neutralizing antibody is a very attractive approach to eliminate gluten toxic peptides (Warny et al., 1999). Moreover, IgG is considered a safe nutritional products similar to milk, thus would not be subject to strict regulatory approval.

b) Transepithelial uptake

The increase of intestinal permeability via opening of the epithelial tight junctions is considered an important contributor to the entering of gluten peptides to the subepithelial lamina propria. *Vibrio cholera* secretes the ZOT toxin which opens the intestinal epithelial tight junctions by binding to the 66-kDa ZOT receptor (Lu et al., 2000; Uzzau et al., 2001). It has been demonstrated that human release a similar paracrine protein called zonulin which acts like ZOT, and that zonulin expression is increased in CD. Thus, an octapeptide (AT-1001) was developed to block the ZOT/zonulin receptor thus preventing the gluten-induced “leaky-gut” (Paterson et al., 2007).

c) Frustrating the adaptive immune response

TG2 inhibitors that target TG2 cross-linking activity that is the basis of toxic gluten modification have been developed (Choi et al., 2005; Lai et al., 1998; Siegel and Khosla, 2007; Watts et al., 2005). There are three groups of TG2 inhibitors, they are (1) competitive inhibitors (putrescine, spermidine, histamine, monodansyl cadaverin, cadaverine, 5-pentylamine, fluoresceine, cystamine, and cysteamine) (Jeitner et al., 2005), (2) reversible inhibitors (guanosine triphosphate analogues) (Pardin et al., 2008), and (3) irreversible inhibitors (iodoacetamide, 3-halo-4,5-dihydroisoxazoles, carbobenzyloxy-L-glutaminyl glycine derivatives, 6-diazo-5-oxo-norleucine, 2-[(2-oxopropyl)thio] imidazolium derivatives) (de Macedo et al., 2002; Hausch et al., 2003). The potential ability of TG2 inhibitor treatment is attractive while also considered risky because of the widespread tissue distribution of TG enzymes. TG2 inhibitors with a high affinity thiol binding group were recently developed which display 70- to 225-fold specificity for TG2 over TG1, TG3, TG6, and factor XIII, raising the hopes for increasing safety of its utilization (Pasternack, Hils, Zedira Company, Darmstadt, Germany, personal communication, September 2009).

d) Immune modulation

An innate immune inhibitor decapeptide (QQPQDAVQPF) was isolated from durum wheat and tested in various *in vitro* systems (De Vincenzi et al., 1995; De Vincenzi et al., 1996; De Vincenzi et al., 1997; De Vincenzi et al., 1998; Giovannini et al., 2000; Silano et al., 2007a;

Silano et al., 2007b; Silano et al., 2008). The researchers hypothesized that the decapeptide induced a switch from a Th1 to a Th2 T cell phenotype after down-regulating IFN-γ and up-regulating IL-10 production in intestinal T cells in CD patients (Silano et al., 2008). Some researchers examined modifications of the toxic gliadin peptides to therefore obtain antagonistic peptides (Anderson et al., 2006; Biagi et al., 1999; Kapoerchan et al., 2008; Xia et al., 2006). Modification of the proline residues P38, P39, and P42 of alpha-gliadin p31-43 can abolish immunogenicity[EH6][Office7] (Schuppan 2009).

e) Induction of tolerance to gluten

HLA-DQ2 has a high affinity to gliadin peptides, is the association followed by the activation of CD4+ T cells. To block this process, several peptides also with high affinity to HLA-DQ2 were designed by amino acid substitutions, dimerization, or introduction of aldehyde groups. Modification of α-2-gliadin p57-73 results in partial agonists which significantly inhibit IFN-γ production (Anderson et al., 2006). Moreover, replacement of leucine L11 and L18 residues in the α-gliadin 33-mer with sterically bulky groups keeps high DQ2 affinity but decrease the T-cell recognition (Xia et al., 2006). Also application of azidoproline-containing gluten peptides that can block the effect of a stimulatory α9-gliadin peptide when used at high concentration (Kapoerchan et al., 2008).

Adding *Bifidobacterium* strains to down-regulate the pro-inflammatory [EH8][YC9]effect in CD patients is considered a simple and safe method (Medina et al., 2008). *Bifidobacterium* found significantly lower in fecal samples of active CD children also lower in biopsies when compared to control subjects (Nadal et al., 2007). Specific strains have been investigated for their anti-inflammatory and regulatory properties by inducing IL-10 production and regulating the Th1/Th2 balance (Baba et al., 2008; Medina et al., 2007). Medina et al. found that *B. longum* ES1 and *B. bifidum* ES2 could suppress the Th1 pro-inflammatory characteristic of the CD.

Another approach is the utilization of some parasites. Human or non-human intestinal parasites such as helminths *Trichuris trichura* and *Trichuris suis* can protect animals from developing immune-mediated diseases such as colitis, reactive airway disease, encephalitis, and diabetes. Clinical trials show that the administration of helminthes can reduce disease activity in patients with ulcerative colitis or Crohn's disease (Elliott et al., 2007; Summers et al., 2005a; Summers et al., 2005b). The rationale of these approaches is that the increased prevalence of autoimmune disorders in developed countries is closely paralleled by absence of intestinal parasites (Sollid and Khosla, 2011).

1.2 Gluten degrading enzymes

The use of nonhuman proteases for gluten detoxification was conducted in the late 1950s (Krainick and Mohn, 1959). Recently two types of enzyme are appealing to treat CD via oral therapy by cleaving large peptides. One is a proline-specific endopeptidase PEP (prolyl endopeptidase) (Hausch et al., 2002; Marti et al., 2005; Piper et al., 2004; Pyle et al., 2005; Shan et al., 2002) and the other one is a glutamin-specific cysteine protease EP-B2.

1.2.1. Prolyl endopeptidases (PEP)

It has been shown in many researches that PEP can cleave proline-rich gluten peptides. After cleavage, two shorter peptides were generated including a new amino and a carboxyl terminus. They could be further digested by brush-border aminopeptidases and carboxypeptidases at the intestinal surface, thus the immunotoxic property from gluten was eliminated. PEP can be produced from different bacterial strains. Dariusz et al. (2006) investigated the newly discovered PEP from fungi *Aspergillus niger* (AN-PEP) (Edens et al., 2005). AN-PEP is a member of the serine peptidase family S28 and shares more sequence homology with lysosomal Pro-X carboxypeptidase and dipeptidyl peptidase II than with prolyl oligopeptidases. It has a good property that it works optimally at 4-5 pH, and remains stable at pH 2, which is resistant to the low pH condition in human stomach. Also this AN-PEP is completely resistant to gastric pepsin. Therefore it can arrive the small intestine successfully to degrade the T cell stimulatory peptide as well as intact gluten molecules. Matthew Siegel et al. (Siegel et al., 2006) compared the two promising PEPs from *Flavobacterium meningosepticum* (FM) and *Myxococcus xanthus* (MX). They found that FM-PEP showed a general improvement over MX-PEP especially in targeting the immunodominant α2 epitope.

Prolyl endopeptidase (PEP) expressed in various microorganisms such as *Flavobacterium meningosepticum*, *Sphingomonas capsulatae*, *Myxococcus xanthus*, and *Aspergillus niger* which can cleave the immunotoxic large gliadin peptides like the 33-mer (Cornell et al., 2005; Marti et al., 2005; Mitea et al., 2008; Pyle et al., 2005; Stepniak et al., 2006). However, the effectiveness of PEP is restricted on the length of their substrates (Fulop et al., 2000; Shan et al., 2004), the long time consuming to completely hydrolyze the gliadin peptides, and their best activity at near neutral pH (Cerf-Bensussan et al., 2007; Matysiak-Budnik et al., 2005). PEP from *Aspergillus niger* is active at low pH (~pH 2), has a higher activity than that from *F. meningosepticum*, thus is favorable to be applied to cleave peptidase-resistant gliadins during gastric transport (Mitea et al., 2008; Stepniak et al., 2006). Furthermore, the combination of PEP from *S. capsulatawith* or *A. niger* and the barley-derived enzyme endoprotease B2 can effectively digest whole wheat gluten *in vitro* and *in vivo* (Gass et al., 2006; Gass et al., 2007).

1.2.2. Endoprotease B2 (EP-B2)

EP-B2 is secreted as a proenzyme (proEP-B2) into the acidic endosperm of germinating barley seeds, where it is autoactivated quickly to its mature form in conditions that are similar to that found in the stomach (Jacobsen and Varner, 1967; Koehler and Ho, 1990; Mikkonen et al., 1996). Mature EP-B2 is stable and active over a wide pH range and efficiently digests α2-gliadin which is known for its immunotoxicity (Bethune et al., 2006).

1.2.3. PEP and EP-B2 mixture (ALV003)

A potential concern was that PEP would not be able to detoxify gluten rapid enough in the duodenum (Koning and Vader, 2003; Matysiak-Budnik et al., 2005), and the effectiveness of PEP might be limited by its substrate chain length restrictions. It was considered that the PEP can be enhanced by the administration of EP-B2 (Bethune et al., 2006). According to Siegel (Siegel et al., 2006), at a gluten: EP-B2: FM-PEP weight ratio 75:3:1, grocery store gluten is fully detoxified within 10 min of stimulated duodenal conditions, as judged by chromatographic analysis, biopsy-derived T cell proliferation assays, and a commercial anti-gluten antibody test. Therefore this combination of PEP and EP-B2 could be a suitable oral therapy of CD. A mixture of both enzymes has reached clinical trials and is produced by Alvine Pharmaceuticals as ALV003.

1.3 Gluten-degrading resident bacteria

1.3.1. Non oral gluten-degrading resident bacteria

In the past few years, a new source of gluten-degrading enzymes has been explores, namely the bacteria naturally colonizing the human digestive tract, starting in the oral cavity (Caminero et al., 2014; Helmerhorst et al., 2010; Zamakhchari et al., 2011) had isolated 144 strains belonging to 35 bacterial species that could be involved in gluten hydrolysis in the human intestine. They found that most of the strains were classified within the phylum *Firmicutis*, mainly from the genera *Lactobacillus*, *Streptococcus*, *Staphyloccus* and *Clostridium*. Some of the isolates were classified within the phylum *Actinobacteria*, mostly from the genus *Bifidobacterium*. Only 12% of the isolates were gram negative bacteria from the phylum *Proteobacteria*. Moreover, they found that several bacterial strains belonging to 11 bacterial species have the ability to hydrolyze the 33-mer peptide. Some bacteria, such as *Lactobacillus mucosae*, *L. rhamnosus*, and *Clostridium botulinum/sporogenes* have a higher hydrolyzing activity than other bacteria like *Enterococcus faecalis* or *Bacillus licheniformis,* even the latters can partly hydrolyze the 33-mer.

Recently, many sourdough lactic acid bacteria were tested for proteolytic activity (Di Cagno et al., 2002) and some *lactobacilli* was selected and used for further studies. They had specialized peptidases ability to digest pro-rich peptides, including 33-mer.

VSL#3 (VSL Pharmaceuticals, Gaithesburg, MD) is a highly concentrated (ca. 5×10^{11} cells/g) mixture of lactic acid and bifido-bacteria which has been discovered to colonize the intestine and to promote several beneficial effects for the host (Campieri and Gionchetti, 1999; Gionchetti et al., 2000; Kaur et al., 2002). From De Angelis et al. (De Angelis et al., 2006), they found that 10^9 cells/g of VSL#3 could hydrolyze totally the 33-mer epitope (750 ppm) after 24 hr of incubation at 37^oC. A considerable amount (150 ppm) of 33-mer was hydrolyzed after 4 hr of treatment by VSL#3. It was indicated that the gliadins treated with VSL#3 decreased the activation of the zonulin release, cytoskeleton reorganization, and intestinal permeability compared to non-hydrolyzed gliadins. Also, they found that the gliadins remained after treatment of VSL#3 had very low or absent toxicity. However, the hydrolyzing ability was lost when individual strains which composed of it were tested, indicating that no single strain contains the total portfolio of peptidases required to degrade pro-rich polypeptides. The utilization of VSL#3 during food processing to produce pre-digested and tolerated gliadins could be promising.

Gallo (Gallo et al., 2005) purified partially an X-prolyl dipeptidyl aminopeptidase (PepX) from sourdough *lactobacillus sanfranciscensis* and combined it with general aminopeptidase type N, and found the 33-mer peptide (0.2 mM) was completely hydrolyzed after 24 hr of incubation at 30^{o}C.

Recently, Wei et al. (Wei et al., 2015) isolated and identified some gluten-degrading and aciduric microorganisms from human feces, and all the 43 strains are belonged to one species, *Pseudomonas aeruginosa*. *P. aeruginosa* is a gram negative bacteria and a member of the proteobacteria representing <10% of the gut colonizers, of which 90% are *bacteroidetes* and *firmicutes* species (Turnbaugh et al., 2007). The enzyme from *P. aeruginosa* was isolated representing pseudolysin, or lasB, which is active in gel at pH 2.0. It shows a remarkable ability to cleave major immunogenic gluten peptides, such as 33-mer, at pH 4.0 and 7.0, but not at pH 2.0 and 3.0. Enzyme combination from various origins with the specificity from pseudolysin could effectively cleave immunogenic peptides at low pH conditions.

1.3.2. Oral gluten-degrading resident bacteria

Helmerhorst et al. (Helmerhorst et al., 2010) first reported the evidence for gluten-degrading microorganisms inhabiting the upper gasto-intestinal tract. They found the gliadin, 33-

mer, and 26-mer peptide were proteolytically degraded at a wide pH range (pH 3-10). The results indicated that the oral microorganisms are a viable source of novel enzymes. Fernandez-Feo et al. (Fernandez-Feo et al., 2013) isolated human saliva and plague and found some oral microbial species which can cleave immunogenic gluten and/or gluten derived substrates including *Rothia mucilaginosa, Rothia aeria, Actinocyces odontolyticus, Streptococcus mitis, Streptococcus sp., Neisseria mucosa*, *Capnocytophaga sputigen*, and they present activities at neutral or near-neutral pH. Among all these bacterial strains, *Rothia mucilaginosa* and *Rothia aeria* were found to rapidly hydrolyze the para-nitroanilide-derivitized substrates Z-YPQ-pNA and Z-KPQ-pNA, which are the gluten-based substrates. *R. mucilaginosa* and *R. aeria* belong to the *Rothia* genus under the *Actinobacteria* phyla. They are mainly colonizing the oral cavity (Dewhirst et al., 2010); (Yamane, 2010), *R. mucilaginosa*, however, has also been found in the upper respiratory tract and the duodenum (Ou et al., 2009). *R. mucilaginosa* and *R. aeria* were found to degrade gliadin in solution. Approximately 50% of the added gliadin amount (250 µg/ml) was degraded in ~30 min. Rapid degradation of the 33-mer and 26-mer by *R. aeria* was noted over 2 hr time interval. Also, the *R. aeria* showed good activity at a wide range of pH from 4 to 10. At pH 3, *R. aeria* still showed activity, although weaker than at pH 7 (Zamakhchari et al., 2011) At pH 2, no activity from *R. aeria* was noted and the activity could not be reactivated upon transfer to neutral pH values. Tian et al. (Tian et al., 2014) investigated the natural microbial inhabitant of the oral cavity, *R. mucilaginosa*, the potential capability to hydrolyze the gliadin 33-mer and 26-mer peptides. They found that the incubation of gliadins with *R. mucilaginosa* cells reduced R5 antibody binding by 20, 82, and 97% after 30 min, 2 hr, and 5 hr, respectively, at pH 7. Although the elimination of epitopes in the 33-mer was less effective than in the 26-mer, the mass spectrometer showed that *R. mucilaginosa* hydrolyzed all the three major immunogenic epitopes comprised in the 33-mer.

1.4. Rothia aeria

R. aeria was first isolated from the air in the Russian space laboratory Mir in 1997 and the taxonomic status was investigated by 16S rDNA sequences in 2004 (Li et al., 2004). This gram positive strain grows well under aerobic conditions on BHI agar plate. The cells appear coccoid, cocco-bacillary or filamentous. Young colonies are white and smooth; mature colonies are rough and dry and adhere to the agar medium. *R. aeria* showed the following common biochemical characteristics with *Rothia dentocariosa* and *Rothia mucilaginosa*: positive for nitrate reduction, pyrazinamidase, alanine-phenylalanine-proline arylamidase, α-glucosidase and β-glucosidase activities, hydrolysis of aesculin and acid production from glucose, maltose and sucrose; negative for β-glucuronidase, β-galactosidase, N-acetyl-β-glucosaminidase and urease activities and production of acid from ribose, xylose, mannitol, lactose and glycogen. The exploitation of *R. aeria* is attractive because it belongs to the normal flora in oral cavity where the entrance of the digestive system is and can be a novel source for therapeutic gluten degrading enzymes.

2. AIMS OF STUDY[gw10][Office11]

Celiac disease is a T-cell mediated-inflammatory disorder of the small intestine precipitated by gluten ingestion. Gluten can be effectively degraded by *R. aeria*, a natural resident oral microbe. In order to develop *R. aeria* as a first probiotic for celiac disease, it is of significance to investigate the efficacy of detoxify gluten *in vivo.* As an initial step, we investigated the optimal conditions to obtain high yield of gluten degrading activity.

Specific aims of this study:

1. To test the effect of strength and carbon source of bacterial growth media as well as incubation time and temperature on the gluten-degrading enzyme expression of *R. aeria in vitro*.
1. To determine the ratio of *R. aeria* to gluten that required to achieve digestion within 1 hr to 2 hr, the residence time of foods in the stomach/upper intestines.

3. MATERIALS AND METHODS

3.1. Growth of *Rothia aeria* in different conditions

a) Broth strength: *R. aeria* was cultured from a glycerol stock on *Brucella* agar plates (*Brucella* with vitamin H & K, A30, Hardy Diagnostics, CA, USA) and incubated at 37^{o}C for 24-48 hr.

BHI broth (100%) was prepared by adding 18.5g of BHI powder (BBL™ Brain Heart Infusion, BD) to 500 ml of MilliQ water in a 1000 ml flask. After autoclaving for 45 min and cooling, several colonies of *R. aeria* were transferred from the plate with the sterile inoculation loop into the bottle. Different BHI broth strengths (20% and 4%) were prepared to compare the effect of it on enzyme activity. The total incubation was 24 hr at 37^{0}C, and the enzyme activity of each bottle was measured every 10 min for 3 hr. The enzyme activity was measured by cleavage of suc-AAPF-pNA.

b) Carbon sources: A chemical defined media M9 broth was prepared by adding 5.64 g of M9 minimal salts (Sigma, MO, USA) to 500 ml of MilliQ water in 4 1000 ml flasks. 2% of glucose, succinate, glycerol, and casein were added into each flask, respectively. After autoclaving for 45 min and cooling, several colonies of *R. aeria* were transferred from the plate with the sterile inoculation loop into these 4 bottles. Bottles of M9+2% glucose, M9+2% succinate, M9+2% glycerol were incubated for 7 days. The bottle of M9+2% casein was incubated for 24 hr. All the bottles were incubated at 37^{0}C. The enzyme activity of each bottle was measured every 10 min for 3 hr by cleavage of suc-AAPF-pNA.

c) Temperature: BHI broth (20%) was prepared by adding 3.7g of BHI powder (BBL™ Brain Heart Infusion, BD) to 500 ml of MilliQ water in a 1000 ml flask. After autoclaving for 45 min and cooling, several colonies of *R. aeria* were transferred from the plate with the sterile inoculation loop into 2 bottles. One bottle was incubated at 37^0C and the other was at 28^0C for 96 hr, and the enzyme activity of each bottle was measured every 10 min for 2 hr. The enzyme activity was measured by cleavage of suc-AAPF-pNA.

d) Incubation time: To determine the generation time (doubling time), aliquots (around 2.5 ml) of each *R. aeria* culture were transferred to two bottles (250 ml/each) of fresh 100% BHI to get the same initial OD. Aliquots (1 ml) of the culture were taken at time points from 0-48 hr with 2 hr intervals, and the bacterial optical density at wavelength 620nm (OD_{620}nm) were determined with a spectrophotometer (Genios Plus, Phoenix). The number of CFU/ml was determined as described below in Section 4. The bacterial generation time was calculated with a formula: G (generation time) = t (minutes or hours) / 3.3 log b/B (number of generations), where b = the concentration of bacteria (or CFU/ml) at the end of the selected time interval and B = the concentration of bacteria (or CFU/ml) at the beginning of the time interval (http://textbookofbacteriology.net/growth_3.html). The interval chosen for these calculations was 6-12 hr in experiment 1 and 12-18 hr in experiment 2[EH12][Office13].

3.2. Enzyme activity Assay

R. aeria cells harvested from the BHI culture or the *Brucella* agar plate were suspended in PBS (pH 7.0) and adjusted to an OD_{620} 0.6. Aliquots of 200 µl from the suspensions were added in triplicate to a 96-well flat bottom microtiter plate (Costar 3596, Corning, NY, USA) containing 4 µl of 10 mM para-nitroanilide-derivatized enzyme substrates Suc-AAPF-pNA (final concentration 200µM). This substrate is measuring subtilisin enzyme activity, the main enzyme produced by the *Rothia* bacteria (Wei et al., 2015). Controls were suspensions without added substrate. Substrate hydrolysis was monitored by detecting the change in absorbance at 405 nm for 3 hr[EH14][Office15] every 10 min at 37^{o}C using a spectrophotometer.

3.3. *Rothia aeria* batch cultivation

Rothia aeria was cultured from a glycerol stock on *Brucella* agar plates (*Brucella* with vitamin H & K, A30, Hardy Diagnostics, CA, USA) and incubated at 37^{o}C for 24-48 hr.

BHI broth (100%) was prepared by adding 9.25g of BHI powder (BBL^{TM} Brain Heart Infusion, BD) to 250 ml of MilliQ water in a 500 ml flask. After autoclaving for 45 min and cooling, several colonies of *R. aeria* were transferred from the plate with the sterile inoculation loop into 2 bottles (250 ml/each).

After incubation shaking (120 rpm) on a platform shaking machine (3D Rocker, model 130, Denville Scientific INC, USA) at 37^{o}C for 24 to 48 hr. The *R. aeria* culture was verified to be pure by plating on *Brucella* agar and visual inspection of colony morphology. To obtain certain amount of *R. aeria* cells, the cells were cultured in BHI under the same conditions in 2-liter bottles and were harvested by centrifugation for 30 min at 4^{o}C, 17000 g (9700 rpm), using a rotor F10-6x500y and a Sorvall RC-6 PLUS Superspeed Centrifuge machine (Thermo Scientific, USA). After centrifugation, the supernatant was removed and cells were washed twice with sterile PBS. After the second wash, the cells were re-suspended in 50 ml PBS and the OD_{620} was adjusted to be around 50, stored in -80^{0}C.

3.4. Viability of *R. aeria* batch cultures

R. aeria cells were suspended in PBS at around OD_{620}= 50. A 10 fold serial dilution series[EH16][Office17] was prepared in 1 ml Eppendorf tubes as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions in duplicate. Aliquots of 50 µl of each of the suspensions from 10^{-3} through 10^{-8} were plated onto *Brucella* agar plates. After incubation at 37°C for 24-48 hr, images were taken using the scanner (G: BOX, SYNGENE, Cambridge, UK), and colonies were counted using the GeneTools software (SYNGENE, Cambridge, UK).

3.5. Lyophilization of cells

R. aeria suspensions from the batch cultures cultured for 16, 24, and 66 hr yield typically per 2-3 L culture a suspension of 50 ml with an OD_{620}=50. The suspension was aliquoted into 1, 2, or 3 ml aliquots in 2 ml Eppendorf tubes [gw18][Office19]and centrifuged (Eppendorf 5424, Hamburg, Germany) at 21,130 x g (15,000 rpm) for 5 min. The supernatant was removed and cells in pellet were dried using a Speedvac. In subsequent experiments, the suspensions were not aliquoted, but the about 50 ml in the 50 ml tube was directly centrifuged, the supernatant removed and the pellet dried in a lyophilizer (FM 25 EL, Virtis, NY, USA). All dried cell pellets were stored in a -80°C freezer.

3.6. Gliadin hydrolysis in gel (gliadin zymography)

Bacterial cells of *R. aeria* were harvested from *Brucella* agar plate or BHI broth after incubation at 37°C for 48 hr and suspended in PBS solution at pH 7.0 with OD_{620} 5.0. Aliquots of 150 µl were centrifuged at 21,130 x g (15,000 rpm) using an Eppendorf centrifuge for 5 min and the cells were re-suspended in equal 25 µl of 2x Zymogram sample buffer containing 0.125M Tris-

HCl, 20% glycerol, 20% SDS, and 0.005% bromophenol blue to each sample. The sample was loaded onto a 6% gliadin zymogram gel as described (Fernandez-Feo, 2013) and electrophoresis was performed at a constant voltage of 100V in the 4^{o}C in the cold room for around 2 hr. The gels were re-natured with 100 ml of renaturing buffer (Invitrogen Novex Zymogram) for 30 min, which was repeated once; then washed with developing buffer for 20 min (Invitrogen Novex Zymogram) for 2 times. After incubation in developing buffer at 37^{o}C for 48 hr, the gel was stained overnight with staining solution containing 0.1% Coomassie blue, 40% methanol and 10% acetic acid. To visualize protein bands, the gel was de-stained with the solution of 40% methanol and 10% acetic acid. The gels were scanned using a scanner (G: BOX, SYNGENE, Cambridge, UK).

3.7. Gliadin hydrolysis in solution

Gliadin solution (5 mg/ml) was prepared by dissolving 5 mg of gliadin (Sigma #G3375-25G) in 1 ml of 60% ethanol and shaking at room temperature for 30 min. Bacterial suspension was prepared by suspending *R. aeria* cells in saliva ion buffer (SIB) to $OD_{620=}$1.0 or specific indication. The experiment was performed in 3 groups: gliadins incubated with *R. aeria*, as a test group; the gliadin only or the bacterium only as controls. All the samples were incubated at 37^{o}C and aliquots of 100 µl of each sample were taken at the time points of 0 hr, 0.5 hr, 1 hr, 2 hr, and 4 hr, and boiled at 100^{o}C for 10 min to stop the enzyme reaction. The samples were lyophilized with SpeedVac machine (SpeedVac Plus SC110 A, Savant, NY, USA) for analysis by SDS-PAGE.

The lyophilized samples were re-suspended with 24 µl MilliQ water and 8µl of 4x Bis-Tris w/DDT sample buffer. After boiling for 5 min, the samples were loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Novex). A 5 µl of aliquot of a protein standard (Protein^{TM} All Blue Standards, BIO-RAD, USA) was loaded to the first lane as the molecular weight markers.

Electrophoresis was performed at a constant voltage of 120 V for about 1 hr at room temperature. The gel was stained with 0.1% Coomassie blue stain in 40% methanol and 10% acetic acid overnight and de-stained with the same solution without dye.

4. RESULTS

4.1. Effect of different conditions on bacterial growth

The OD_{620} of 24 hr *R. aeria* culture in 4%, 20%, and 100% BHI were 0.240, 0.932, and 2.033, respectively. No bacterial growth was observed in M9 broth +2% glucose, succinate, or glycerol but grew well in M9 broth +2% casein with OD_{620} 19.54.

4.2. Growth curve and generation time of *Rothia aeria*

The growth curves obtained in experiment 1 and experiment 2 are shown in[EH20][Office21] Figures 1A and 1B, resp. The bacteria grew slow at the early stage (0-6 hr for experiment 1 and 0-12 hr for experiment 2). The OD increased from 0.1341 to 6.098 during the 6-28 hr interval in experiment 1 (Fig 1A), and from 0.2226 to 3.36575 during the 12-24 hr interval in experiment 2 (Fig 1B), representing the logarithmic phase. After 24-28 hr the bacterial growth reached a stable level with OD 5.5 – 6.0 (Exp.1), representation the stationary phase or 2.6-3.6 (Exp.2). The logarithmic plots of time against the log of the CFU/ml are shown in Figure 1C (Exp.1) and D (Exp.2). The log phase, read from the linear plots was occurring from[EH22][Office23] 6 hr to 12 hr in Exp.1 and from 12 hr to 18 hr in Exp.2. The generation time (doubling time) of *R. aeria* was 1.79 hr (6/ 3.3 x 1.0134=1.7941) or 2.15 hrs (6/ 3.3 x 0.8442= 2.1537) for Exp.1 or Exp.2, respectively.

[gw24]

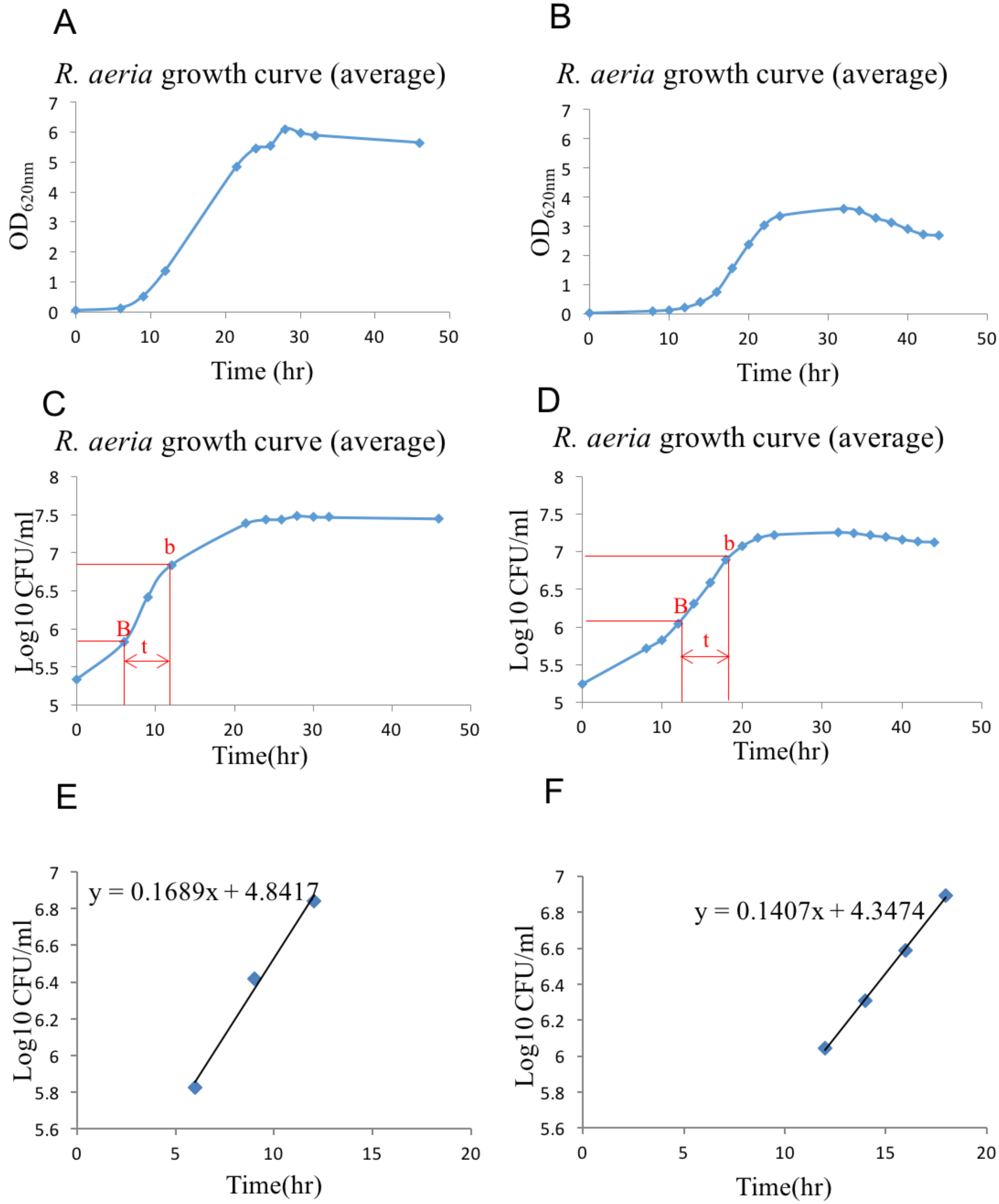


Figure 1. Growth curves of two cultures of *R. aeria*. Two experiments were carried out, Experiment 1 (A, C, E), Experiment 2 (B, D, F). *R. aeria* was incubated in 100% BHI at 37°C. The average OD_{620nm} of both cultures was measured over time (A, B). From the logarithmic plots (C, D) the doubling time was calculated. The slope of the doubling time (E, F).

[Office25][gw26][Office27]

4.3. Bacterial enzyme expression

Broth strength: *R. aeria* cultured in BHI broth strength 4%, 20%, and 100% exhibited significant different enzyme activity expression (Figure 2A). The enzyme activity in proportional to the strength of BHI. 100% BHI showed the highest and 4% BHI the lowest with initial enzyme reaction rates 0.22 and 0.1 AU/min, respectively, 20% BHI was in between with the rate 0.12AU/min (Figure 2B).

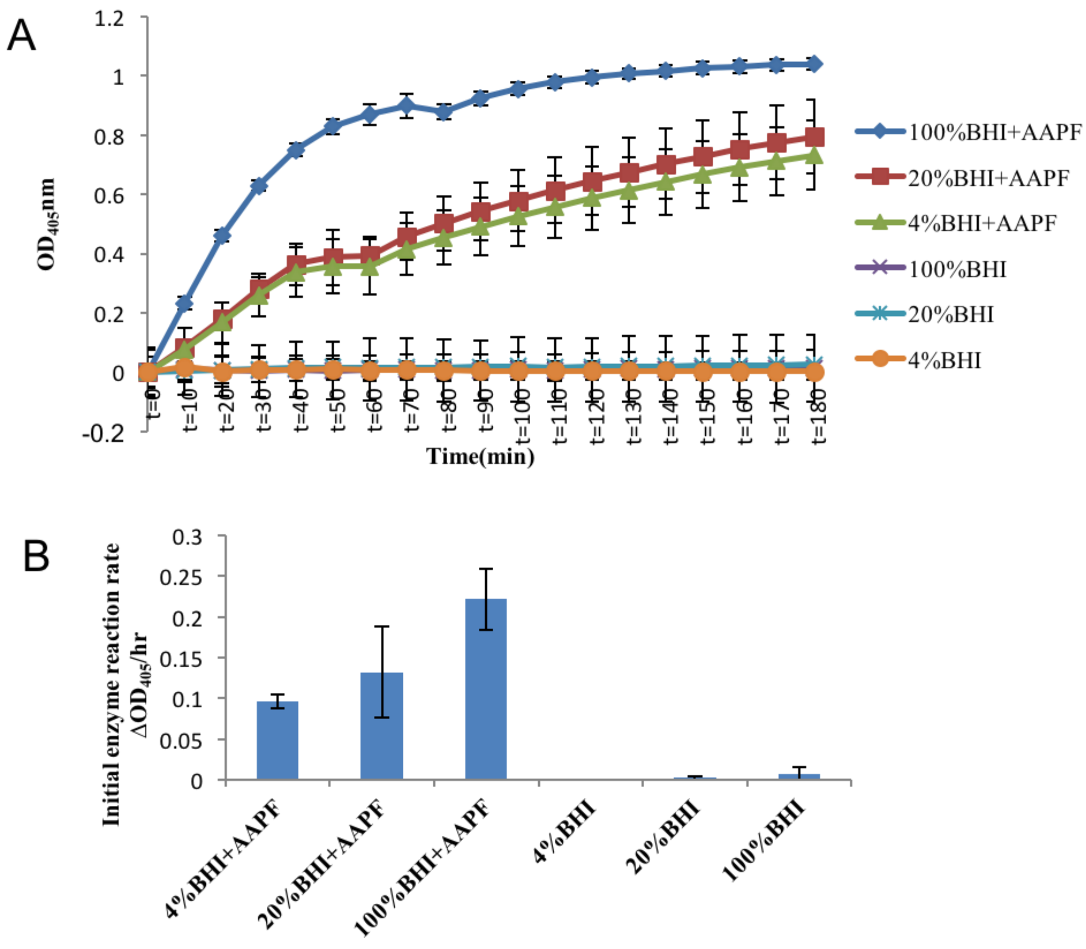


Figure 2. The effect of broth strength on *R. aeria* enzyme activities monitored by cleavage of suc-AAPF-pNA. A: enzyme reaction plot in each condition; B: the initial enzyme reaction rate derived from the enzyme reaction plot. 100 % BHI is higher than 20% and 4% BHI.

As shown in Figure 3, the enzyme activities in M9+2% casein were low (0.035 AU/min), which is not in proportional to the OD of *R. aeria* culture.

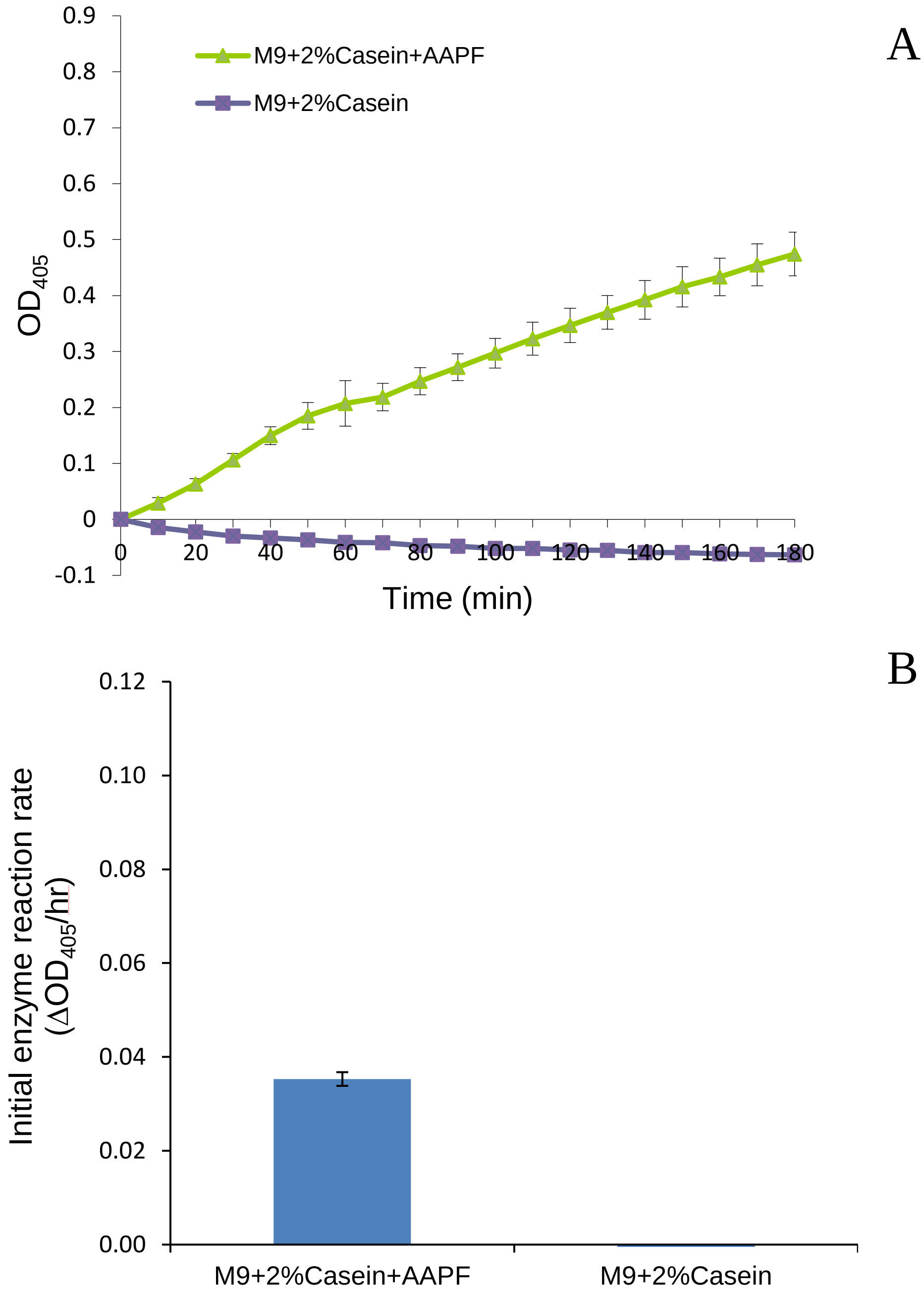


Figure 3. The effect of carbon sources on *R. aeria* enzyme activities monitored by cleavage of suc-AAPF-pNA. A: enzyme reaction plot in each condition; B: the initial enzyme reaction rate derived from the enzyme reaction plot.

R. aeria showed higher activity after cultivated in 100% BHI at 37°C than at 28°C as shown in Figure 4A. The initial enzyme reaction rate was 0.11 AU/min at 37^{0}C, and 0.07 AU/min at 28^{0}C as shown in Figure 4B.

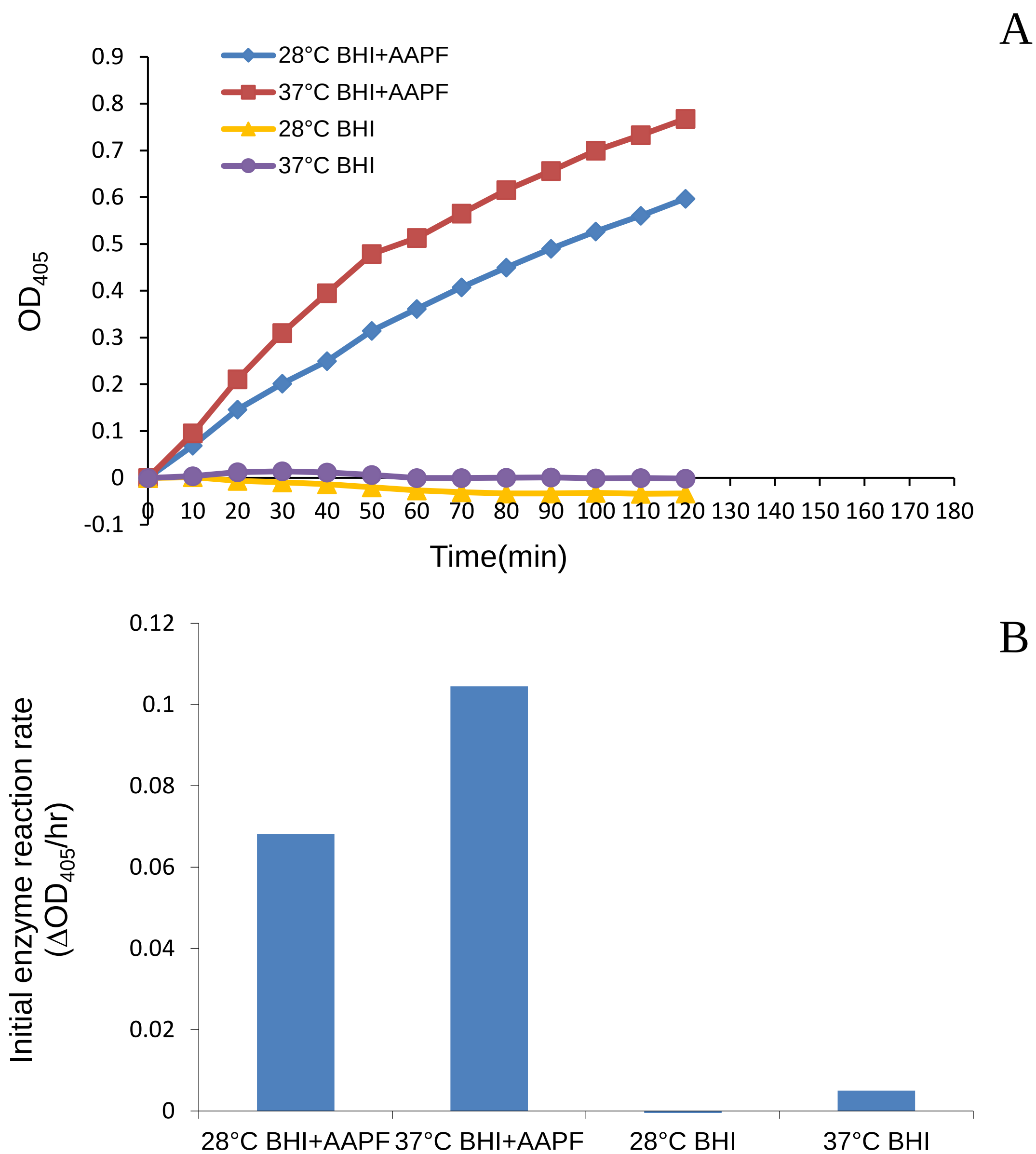


Figure 4. The effect of incubation temperature on *R. aeria* enzyme activities monitored by cleavage of suc-AAPF-pNA. A: enzyme reaction plot in each condition; B: the initial enzyme reaction rate derived from the enzyme reaction plot.

[gw28][gw29][Office30]4.4. Enzyme activity of *R. aeria* throughout the growth phase

To assess the initial enzyme activity at different incubation time, two experiments were performed by incubating *R. aeria* in two bottles (1 L/bottle) of 100% BHI. The enzyme activity was higher in log phase than stationary phase in both experiments (Figure 5A and 5B) with the average rates 0.3 AU/min at 12 hr of experiment A, or 0.75 AU/min, at 14 hr of experiment B (Figures 5C and 5D[EH31])[Office32].

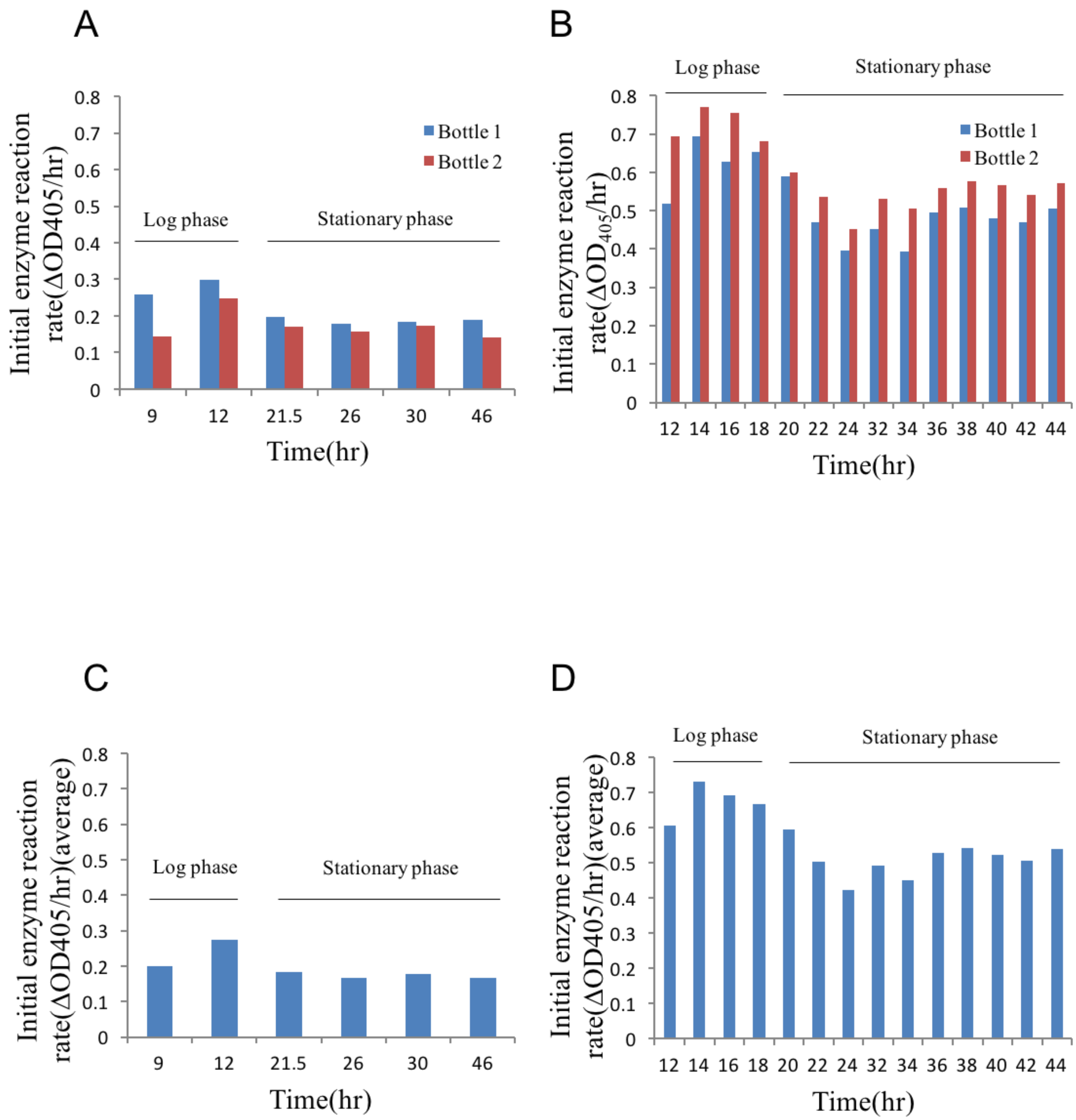
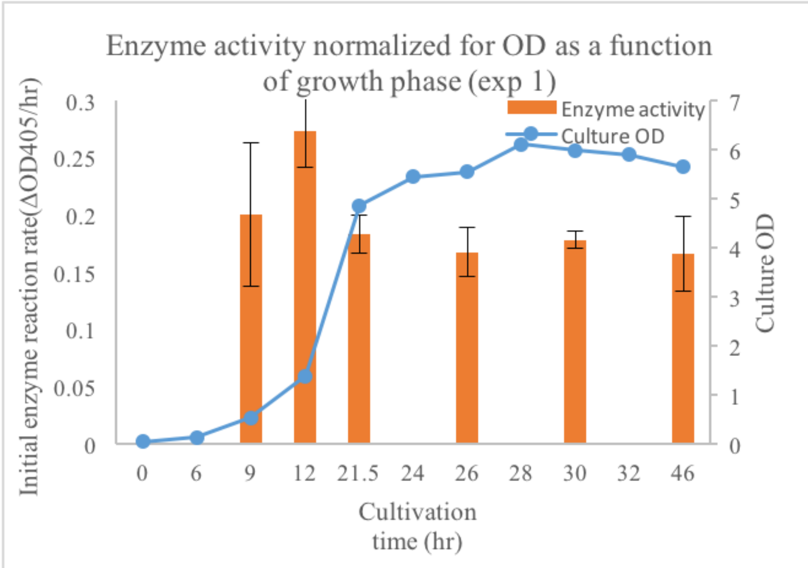


Figure 5. Enzyme activity of *R. aeria*. Two experiments were carried out, experiment 1 (A, C), experiment 2 (B, D). The initial enzyme reaction rates were measured at specific time intervals from 2 bottles during total observed time (A, B). The average initial enzyme reaction rate of the two bottles (C, D).

4.5. Enzyme activity and culture OD as a function of cultivation time

From the same experiments, we also plotted the enzyme activity and culture OD as a function of cultivation time (Figure 6A, experiment 1; 6B, experiment 2). As shown in Figure 6A, the enzyme activity reached to the highest in 12 hr which referred to the log phase and stayed in a stable level in stationary phase. As shown in Figure 6B, the enzyme activity reached to the highest in 14 hr which also referred to the log phase and decrease to a lower level in the stationary phase. From both experiments, the maximum enzyme activity reached earlier (log phase) than bacterial cells as expressed.[gw33][Office34]

A



B

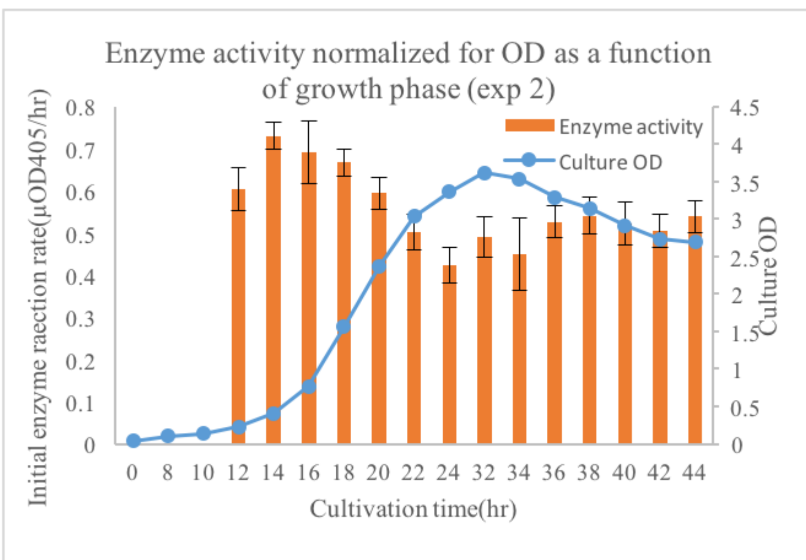


Figure 6. Assessment of *Rothia* enzyme activity as a function of *R. aeria* growth phase. Two experiments were carried out. Experiment 1 (A). Experiment 2 (B**).** *R. aeria* (2 bottles) were incubated in 100% BHI at 37°C. At various time intervals, aliquots were removed, normalized for OD_{620} (0.6) and enzyme activity measured using Suc-AAPF-pNA as the substrate. Constitutive enzyme expression was observed, which was highest in log phase cells.

4.6. The total activity yields as a function of growth

In order to get sufficient amount of enzyme from *R. aeria* cells for further experiments, the total activity the enzyme yielded from the bacterial culture in terms of OD_{620}nm was assessed. As shown in Table 1 and Figure 7, in experiment 2, culture at the time of 20-44 hr (stationary phase), with OD unit 2.5-3.5, the total activities yielded by *R. aeria* were higher from 1.38-1.48; while at time 12-18 hr (log phase), with OD unit 0.19-1.5, the total activities were lower from 0.14 to 1.04. [gw35][Office36]The total activities reached the highest starting at 32 hr, suggesting stationary phase was appropriate time to get higher yield of activity.

Table 1. Total activity yield as function of growth (OD_{620}nm)

time(h)	Activity/OD	OD unit	total activity
12	0.6048	0.2226	0.13786092
14	0.7308	0.40705	0.29925966
16	0.6909	0.77	0.53517816
18	0.6669	1.5609	1.04129766
20	0.5946	2.36775	1.4081046
22	0.5019	3.02915	1.52107335
24	0.4236	3.36575	1.4231613
32	0.4908	3.61825	1.77600795
34	0.45	3.53	1.5929919
36	0.5268	3.29075	1.7367018
38	0.5415	3.13925	1.7042346
40	0.5226	2.913	1.5255738
42	0.5049	2.735	1.3829373
44	0.5391	2.693	1.4528952

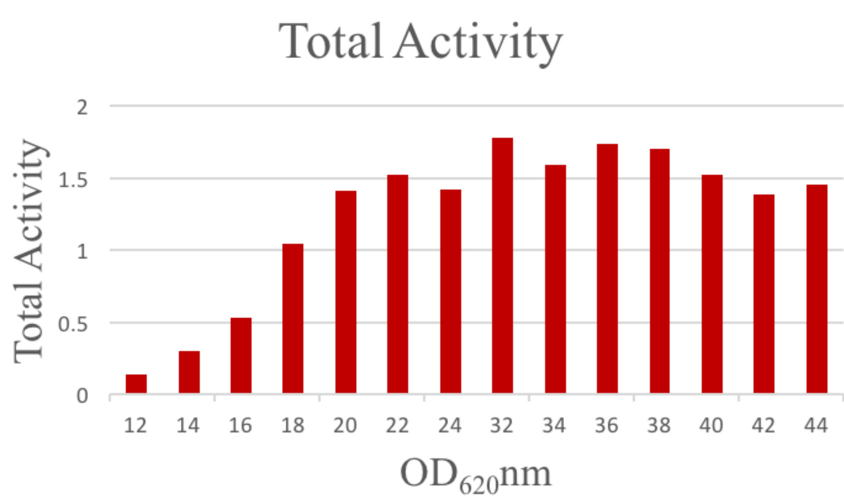


Figure 7. Total activity yield as a function of growth (OD_{620}nm). In experiment 2, two bottles were measured and the average of them was showed. The total activity was calculated from the measurements of the activity/OD and OD unit as shown in Table 1.

[gw37][Office38]In the meantime, the enzyme activities of 30 batches (Table 2) corresponding cultures were also analyzed. As shown in Figure 8, the enzyme activities of the 16 and 24 hr cultures were comparable with the average initial enzyme reaction rates (SD) as 0.121 (0.044) and 0.118 (0.019) AU/min, and were higher than that of 66 hr as 0.077 (0.027) AU/min, indicating early culture (log phase) expressed higher activity than the stationary phase.

[gw39]

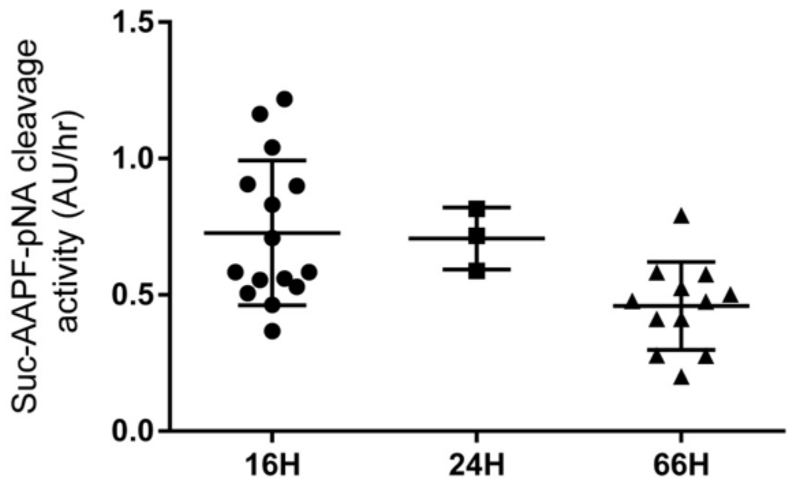


Figure 8. Enzyme activity of *R. aeria.* The bacterial cells in the 30 batches cultured in BHI at 37°C. The enzyme activity (initial rate) was measured in cells cultured for 16, 24, and 66 hr.

4.7. Gliadin Zymography

The purpose of this experiment was to validate the gliadin degradation activity of *R. aeria* by gliadin zymography. As shown in Figure 9, the gliadin conjugated in 6% zymogram gel was hydrolyzed, appeared as white bands in the gel at ~75kDa, by the bacterial cells from both *Brucella* [gw40][Office41]agar plate (Figure 9, lane 2 and 3), and 100% BHI broth (Figure 9, lane 4 and 5).

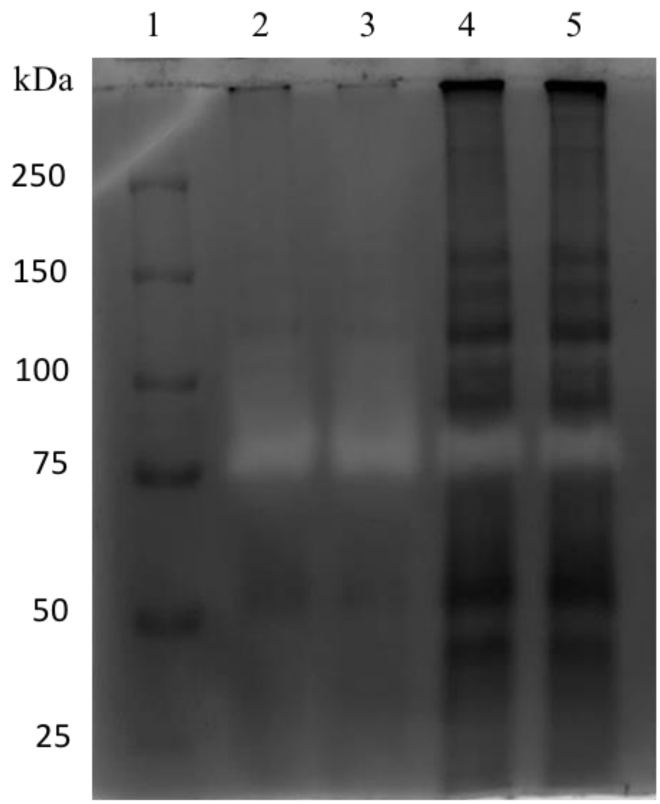


Figure 9. Validation of gliadin-degrading enzyme activity of *R. aeria.* Cells were either harvested from a *Brucella* agar (BA) plate cultured for 48 hr at 37°C with a cotton swab, or cultured in BHI for 48 hr at 37°C. Suspension were prepared in PBS buffer, adjusted to OD_{620} 5.0. Aliquots of 150 µl were centrifuged, and cells re-suspended in 25 µl sample buffer. After electrophoresis, gels were renatured, developed (48 hr), and stained with Coomassie Brilliant Blue to visualize proteins with enzymatic activity. Lane 1, MW std (5 µl); lane 2, *R. aeria* from BA plate 1; lane 3, *R. aeria* from BA plate 2; lane 4, *R. aeria* from BHI culture 1; lane 5, *R. aeria* from BHI culture 2.

4.8. Gliadin hydrolysis in solution

The capability of gliadin degraded by *R. aeria* was also assessed in solution. Mixed gliadin (250 µl/ml) with *R. aeria* of OD_{620}1.0 was incubated for different time points: 0, 0.5, 1, 2, and 4 hours. Aliquots of 100 µl were centrifuged, and cells were re-suspended in 8 µl 4x sample buffer. After electrophoresis, gels were stained with Coomassie blue to visualize proteins with enzymatic activity. As shown the major gliadin band at 37 kDa (arrow) in Figure 10, the gliadins were degraded by *R. aeria* in a time-dependent manner after incubation from 0 to 4 hr, with high degradation when incubated 2 and 4 hr.[gw42] In corresponding to the degradation, the degraded peptide products at 10 kDa appeared in the same way. Similar gliadin hydrolysis was also demonstrated in solution by batches #4, #5, #8, #9, #10, #13, #14, and #15 (Table 2) of *R. aeria* cultures (Figure 11), although there were some varies in the degradation efficiency between the different batches.

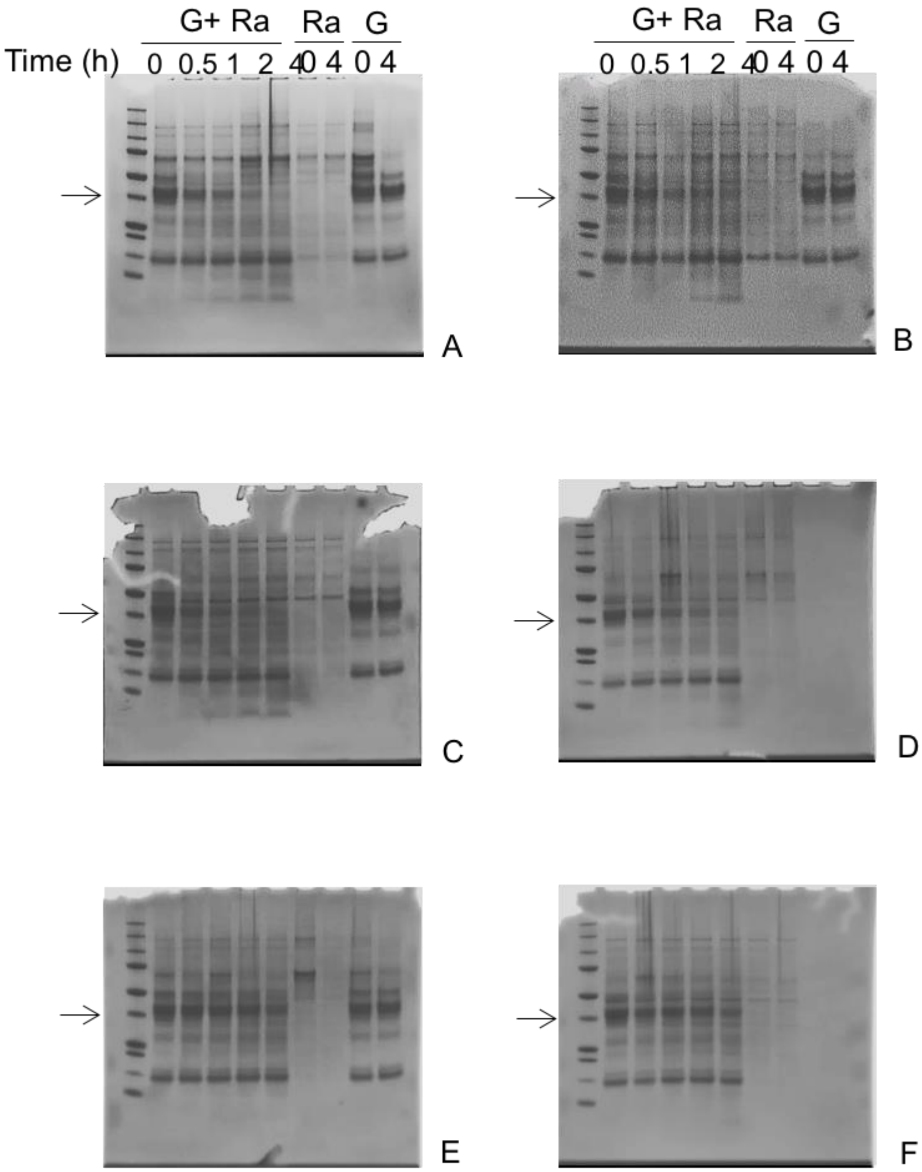


Figure 10. Assessment of gliadin hydrolysis in solution by batches #15-#18 (Table 2) of *R. aeria* cultures. Gliadins (250 µl/ml) were mixed with *R. aeria* of OD_{620}1.0. At t=0, 0.5, 1, 2, and 4 hr aliquots of 100 µl were dried in SpeedVac centrifuge machine, and the pellet was re-suspended in 28 µl 4x Bis-Tris w/DDT sample buffer. After electrophoresis, gels were stained with Coomassie blue to visualize proteins. Lane 1, MW std (5 µl); lane 2, R. aeria+gliadin at 0 hr; lane 3, *R. aeria*+gliadin at 0.5 hr; lane 4, *R. aeria*+gliadin at 1 hr; lane 5, *R. aeria*+gliadin at 2 hr; lane 6, *R. aeria*+gliadin at 4 hr; lane 7, *R. aeria* at 0 hr; lane 8, *R. aeria* at 4 hr; lane 9, gliadin at 0 hr, lane 10, gliadin at 4 hr. Gliadin band was assessed at ~37 kDa (arrow). A, B: R. aeria was from different origin to be compared for the enzyme reaction ability. C: #15 batch 66H E1, D: #16 batch 66H E2, E: #17 batch 66H Ch1, F: #18 batch 66H Ch2. Note: Gliadin control not included in (D)(F).

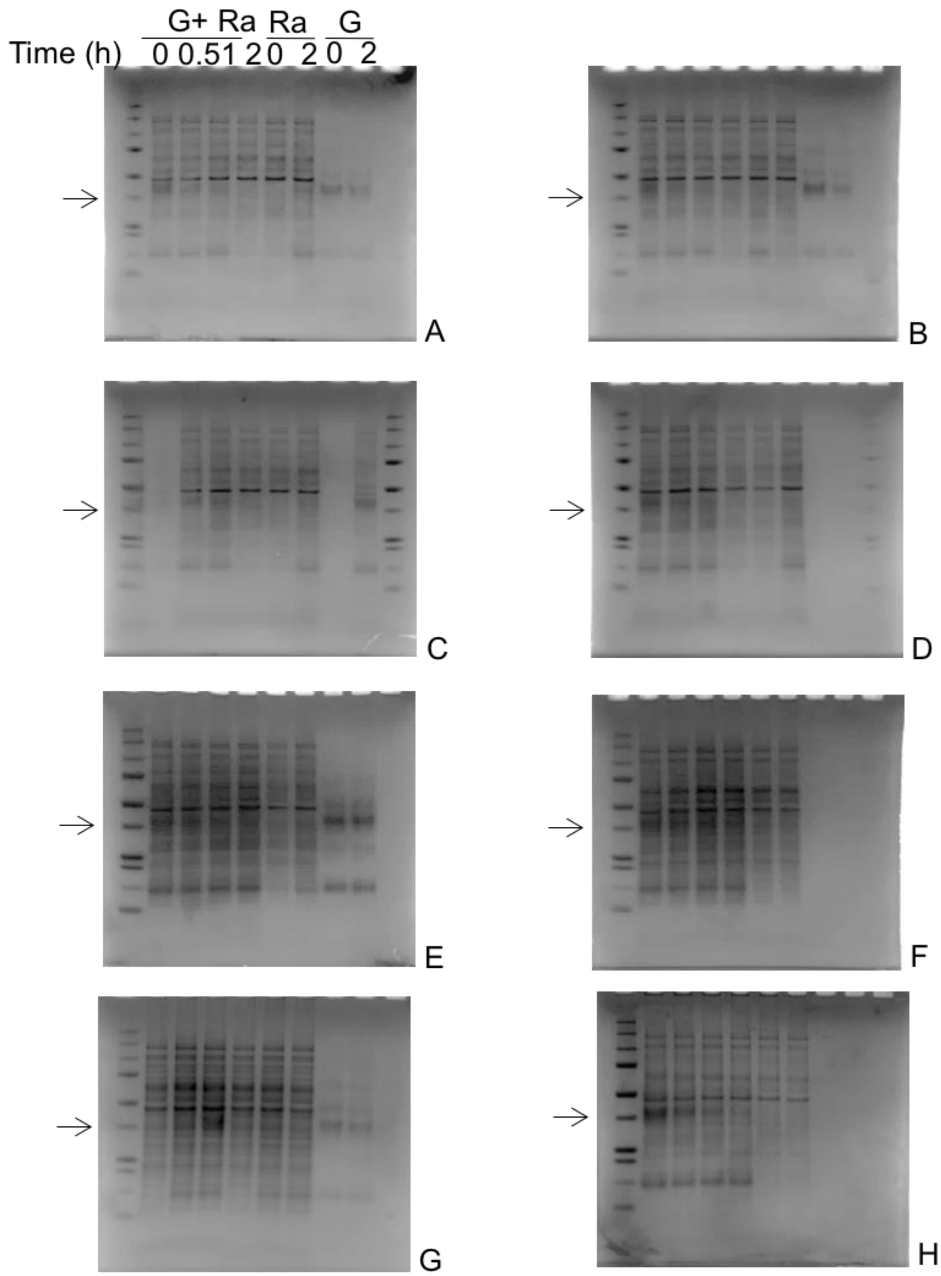


Figure 11. Assessment of gliadin hydrolysis in solution in batches #4, #5, #8, #9, #10, #13, #14, #15 (Table 2) of *R. aeria* cultures. Mixed gliadin (250 µl/ml) with *R. aeria* of OD_{620} 1.0 was incubated for different time points: 0, 0.5, 1, and 2 hr. Aliquots of 100 µl were centrifuged, and cells were re-suspended in 8 µl sample buffer. After electrophoresis, gels were stained with Coomassie blue to visualize proteins with enzymatic activity. Lane 1, MW std (5 µl); lane 2, *R. aeria*+gliadin at 0 hr; lane 3, *R. aeria*+gliadin at 0.5 hr; lane 4, *R. aeria*+gliadin at 1 hr; lane 5, *R. aeria*+gliadin at 2 hr; lane 6, *R. aeria* at 0 hr; lane 7, *R. aeria* at 2 hr; lane 8, gliadin at 0 hr; lane 9, gliadin at 2 hr. Gliadin band was assessed at ~37 kDa (arrow). A: #4 batch 24H, B: #5 batch 16H, C: #8 batch 16H 2L, D: #9 batch 16H 3L, E: #10 batch 16H bottle B, F: #13 batch 16H bottle A, G: #14 batch 16H bottle B, H: #15 batch 66H E1. Note: Gliadin control not included in (D)(F)(H).

4.9. Effect of cell density on gliadin degradation

From this experiment, different gliadin to OD ratios were examined in order to decide which ratio should be applied appropriately to future *in vivo* test. A fixed concentration 250 µg/ml of mixed gliadins was incubated with *R. aeria* at OD_{620} at 1.0, 0.5, or 0.25 at 37^{o}C for 0.5 and 1 hr. The degradation was monitored by SDS-PAGE and major gliadin bands at ~37kDa were assessed. As show in Figure 12, the gliadins were obviously degraded by *R. aeria* at OD=1.0 in 1 hr, while no such degradation were observed by *R. aeria* at OD=0.5 and 0.25. Therefore, the optimal ratio of cell density to gliadin was considered to be 1:250 (OD: µg/ml). [gw43]

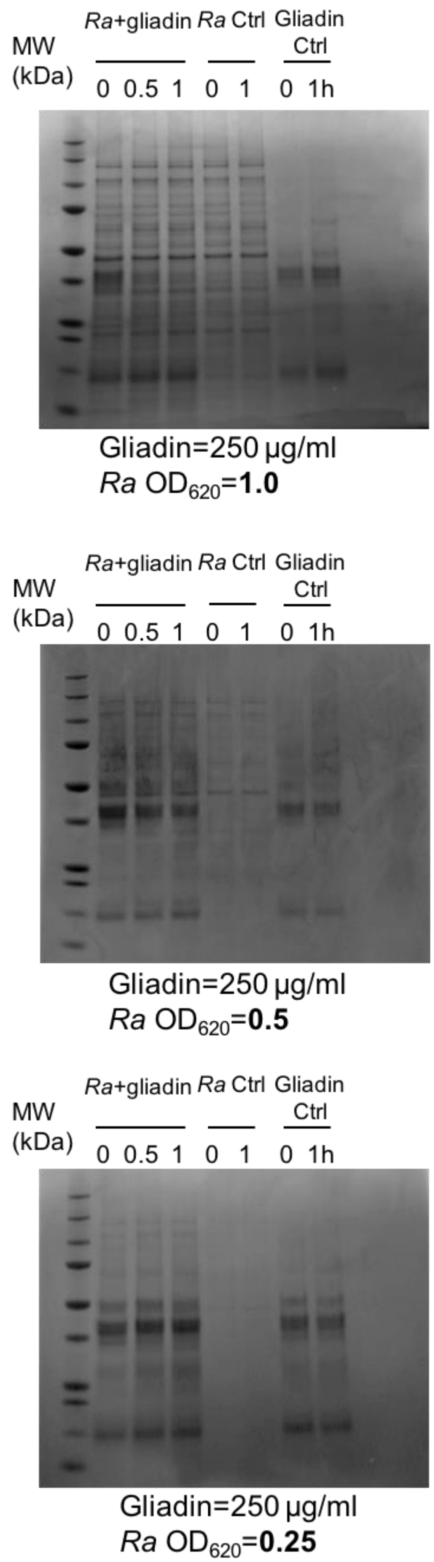


Figure 12. Effect of cell density on gliadin degradation. A fixed concentration of mixed gliadins (250 µg/ml) was incubated with *R. aeria* at OD620 at 1.0 (A), 0.5 (B) or 0.25 (C) at 37°C for 0.5 and 1h. The degradation was monitored by SDS-PAGE (main gliadin band at ~37 kDa).

The information including the amount of viable bacterial cells and activities of 30 batches of *R. aeria* culture were summarized in Table 2. The OD ranged from 78.8 to 5003 with medium 1143. The viability ranged from 4.0 x 10^8 to 7 x 10^{10} CFU with medium 9.25 x 10^9 CFU. The enzyme activity from 0.2004 to 1.2186 OD_{405}/hr with medium 0.5511 AU. All 30 batches will be used for the future *in vivo* and *in vitro* experiments.

Table 2. ***R. aeria*** **in BHI culture information, enzymatic activity and viability[1].**

Batch	Date	Incubation time	Bottle No.	Vial No.	OD each vial	Total OD unit	Each vial CFU	Amount in total CFU	CFU/ml (OD1)	OD_{405}/hr (OD0.6)	Type of origin of Ra
1	111015	24H		25	104.0		$2.6*10^{7}$	$6.6*10^{8}$	$3.9*10^{5}$	0.5886	1 colony
2	101615	24H		10	55.4	554.3	$1.1*10^{8}$	$1.1*10^{9}$	$2.0*10^{6}$	0.7164	half plate
3		16H		18	25.6	460.4	$9.0*10^{7}$	$1.6*10^{9}$	$3.5*10^{6}$	0.5544	half plate
4	102315	24H		13	50.7	659.1	$1.9*10^{8}$	$2.4*10^{9}$	$3.7*10^{6}$	0.8154	half plate
5		16H		10	53.1	531.0	$3.1*10^{8}$	$3.1*10^{9}$	$5.8*10^{6}$	0.9054	half plate
6	110515	16H	A	25	46.3	1158.0	$2.0*10^{8}$	$4.9*10^{9}$	$4.3*10^{6}$	0.5592	whole plate
7		16H	B	34	53.1	1805.7	$2.7*10^{8}$	$9.0*10^{9}$	$5.0*10^{6}$	0.7086	whole plate
8	111215	16H	2L	36	48.2	1734.5	$2.6*10^{8}$	$9.5*10^{9}$	$5.5*10^{6}$	1.041	whole plate
9		16H	3L	20	52.2	1044.0	$8.1*10^{8}$	$1.6*10^{10}$	$1.6*10^{7}$	1.1628	whole plate
10	111315	16H	B	2	46.8	93.6	$2.0*10^{8}$	$4.0*10^{8}$	$4.3*10^{6}$	1.2186	whole plate
11	112015	16H	A	47	106.2	5003.6	$5.0*10^{7}$	$2.4*10^{9}$	$4.8*10^{5}$	0.5298	1 colony
12		16H	B	22	138.2	3559.7	$8.7*10^{7}$	$2.2*10^{9}$	$6.3*10^{5}$	0.5058	1 colony
				5	103.7		$6.5*10^{7}$				
13	112315	16H	A	18	152.6	2950.5	$3.6*10^{9}$	$7.0*10^{10}$	$2.4*10^{7}$	0.831	several colonies
				2	101.7		$2.4*10^{9}$				
14	113015	16H	B	20	101.4	2028.8	$2.8*10^{9}$	$2.8*10^{10}$	$2.8*10^{7}$	0.8994	several colonies
15	120715	66H	E1	22	101.5	2232.3	$3.0*10^{9}$	$6.6*10^{10}$	$3.0*10^{7}$	0.792	several colonies
16		66H	E2	22	86.0	1892.0	$2.0*10^{9}$	$4.4*10^{10}$	$2.4*10^{7}$	0.576(0.6996)	several colonies
17		66H	Ch1	21	147.6	3098.3	$9.9*10^{8}$	$2.1*10^{10}$	$6.7*10^{6}$	0.2004	several colonies
18		66H	Ch2	21	64.3	1350.0	$5.5*10^{8}$	$1.1*10^{10}$	$8.5*10^{6}$	0.2778	several colonies
19	120815	16H	A	13	80.7	1049.4	$9.2*10^{8}$	$1.2*10^{10}$	$1.1*10^{7}$	0.4632	several colonies
20		16H	B	5	114.7	573.7	$3.0*10^{9}$	$1.5*10^{10}$	$2.6*10^{7}$	0.5826(0.543)	several colonies
21	121515	16H	A	24	44.7	1461.8	$1.3*10^{8}$	$3.2*10^{9}$	$3.0*10^{6}$	0.5826	several colonies
22		16H	B	16	106.9	945.1	$2.6*10^{8}$	$4.5*10^{9}$	$2.4*10^{6}$	0.3666	several colonies
				2	71.3	78.8	$1.7*10^{8}$				
23	122115	66H	A	17	59.5	1011.0	$1.3*10^{9}$	$2.2*10^{10}$	$1.1*10^{7}$	0.4122	several colonies
24		66H	B	19	59.4	1128.8	$1.3*10^{9}$	$2.5*10^{10}$	$1.1*10^{7}$	0.4752	several colonies
25		66H	C	18	61.5	1106.8	$9.7*10^{8}$	$1.7*10^{10}$	$7.9*10^{6}$	0.5832(0.7422)	several colonies
26		66H	D	17	58.4	992.0	$2.4*10^{9}$	$4.0*10^{10}$	$2.0*10^{7}$	0.4122	several colonies
27	11216	66H	A	42ml	49.8	2090.8	$7.2*10^{9}$	$7.2*10^{9}$	$3.5*10^{6}$	0.4782	several colonies
28		66H	B	37.5ml	20.7	777.4	$1.5*10^{9}$	$1.5*10^{9}$	$1.9*10^{6}$	0.5256	several colonies
29		66H	C	50ml	23.6	1178.0	$6.6*10^{8}$	$6.6*10^{8}$	$5.6*10^{5}$	0.5022	several colonies
30		66H	D	50ml	27.3	1364.0	$4.4*10^{9}$	$4.4*10^{9}$	$3.2*10^{6}$	0.2772	several colonies

[1]The length of culturing time of the 30 batches were 16, 24, or 66 hr. The enzyme activity was assessed with Suc-AAPF-pNA (200 µM) with a *R. aeria* cell suspension of OD_{620} 0.6. The viability was determined by plating aliquots of 50 µl from a 10-fold serial dilution series ranging from 10^{-3} to 10^{-8}. The stock suspensions were aliquoted in 2 ml portions in Eppendorf tubes, centrifuged, lyophilized and frozen at -80°C for use in *in vitro* and *in vivo* digestion models.

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5. DISCUSSION

Several media can be applied to culture *R. aeria*, and the enzyme expression can be different cultured from them. One is the *Brucella* agar plate (*Brucella* with vitamin H & K, A30, Hardy Diagnostics) with Hemin and vitamin K. It is modification of the formulation given by the American Society of Microbiology. This medium is rich in nutrients and is a non-inhibitory general growth medium for all gram positive and gram negative anaerobic bacteria. The incorporation of Hemin and vitamin K into *Brucella* agar plate enhances the cultivation of some species of anaerobes. The media contains dextrose which provides an energy source; peptones to provide nitrogenous compounds; and yeast extract to supply vitamin B. Sheep blood provides growth factors required by some anaerobic bacteria. In this study, we used *Brucella* agar plate as nutrient-rich medium to obtain the inoculum of *R. aeria*.

Other selections of cultivating media are from brain heart infusion (BHI), such as BHI agar and BHI broth. They may have different effects on enzyme expression. Brain Heart Infusion agar (Becton Dickinson and Company) is a general purpose culture medium which supports the growth of a wide variety of bacterial and fungal species including many types of pathogens. The main ingredients are brain heart infusion, peptic digest of animal tissue, pancreatic digest of casein, sodium chloride, dextrose, disodium phosphate, and agar. The standard preparation is to suspend 52 g of the powder in 1000 ml MilliQ water and mix thoroughly. Heat with frequent agitation and boil 1 min to completely dissolve the powder. The final pH is 7.4. The other BHI medium is Brain Heart Infusion (BBL^{TM} Brain Heart Infusion, BD) broth. It is an enriched non-selective general purpose broth medium that is useful in the cultivation of a wide variety of bacterial and fungal species. The basal medium is an infusion from brain and beef heart, with peptic digest of animal tissue, sodium chloride, dextrose, pancreatic digest of gelatin, and disodium phosphate. The 100% BHI broth was prepared by mixing 37 g of BHI powder with 1000 ml of MilliQ water. In our pilot study, we compared enzyme activities of *R. aeria* cultured in both of these media and no significant difference was noted (data not shown).

According to prior researches, enzyme was higher in starvation condition by using low strength (20%) diluted broth compared to full strength broth. However, in our study, we found the enzyme expression in 100% BHI broth was higher than that from 20% or 4% broth. It appeared starvation did not enhance the enzyme expression activity by *R. aeria* and high nutritional supply was required. Based on the result of bacterial culture (Figure 7), the most total activity yields occurred in the stationary phase which indicting the best harvesting timing of *R. aeria* even though the most enzyme activity occurred in the log phase.

In general, the more cells the more enzyme produce. In this study, the total bacterial enzyme was in proportional to the yield the bacterial cells when *R. aeria* grew in BHI broth. However, it was not observed when the bacterial grew in M9 + 2% casein broth.

M9 mineral medium is a chemical defined medium used to grow *B. subtilis* in the presence of glucose or other variable carbon sources. It contains Na_2HPO_4, KH_2PO_4, NaCl, glucose, $MgSO_4$, $CaCl_2$, biotin, thiamin, and trace elements. Casein is a family of related phosphoproteins, containing a high number of proline residues. It is the most protein found in milk and relatively hydrophobic. In this study, *R. aeria* grew well in M9 + 2% casein medium in 24 hr but showed lower enzyme activity compared to other BHI combinations. This indicates that casein provides the nutrition which is needed by *R. aeria* but does not facilitate high enzyme activity. Therefore, BHI was chosen to be the main cultivating medium because it is the most effective medium to culture the most of bacteria. Recently, Tsuzukibashi (Tsuzukibashi et al., 2017) developed a novel elective medium (ORSM) for isolating oral *Rothia* species. ORSM consists of tryptone, sodium gluconate, Lab-Lemco powder, sodium fluoride, neutral acriflavin, lincomycin, colistin, and agar. The average growth recovery of oral *Rothia* species on ORSM was 96.7% compared with that on BHI-Y agar. ORSM appears to be better for *R. aeria* growth, but it would be interest to further study the enzyme expression with this medium.

Para-nitroanilide-derivatized enzyme substrates Suc-AAPF-pNA (200 µM) is a substrate for alpha-chymotrypsin and fungal chymotrypsin-like serine protease. Enzymatic cleavage of 4-nitroanilide substrates yields 4-nitroaniline which shows yellow color and the changing is monitored by detecting the change in absorbance over time at 405 nm. Similar to Tyr-Pro-Gln (YPQ) and Leu-Pro-Tyr (LPY), Suc-AAPF-pNA can also be hydrolyzed by gliadin degrading enzyme (subtilisins) from *Rothia mucilaginosa* (Wei et al., 2016). Suc-AAPF-pNA is commercially available, more economic and sensitive than YPQ and LPY. In this study, we selected Suc-AAPF-pNA as a substrate to assess the hydrolysis of gliadin immune epitopes. In the enzyme assay, it is important to the standardized bacterial amount to assess the enzyme expression by certain amount of bacterial cells. In the enzyme assay, *R. aeria* concentration was standardized as OD_{620}=0.6, 50% less than the bacterial cells when used YPQ or LPY as substrate.

In agreement with the results of AAPF cleavage activity, the activity of gliadin hydrolysis in gel and in solution was also detected, indicating that *R. aeria* grown under our conditions was effective in degrading gliadin and the produced bacterial cells are valid to be used as a potential probiotic candidate for further study *ex vivo* or *in vivo* in future.

To study the efficacy of *R. aeria* as a probiotic for elimination of gliadin immune epitopes, it is important to know the effective dosage of *R. aeria*. Our data showed the optimal ratio of *R. aeria* to gliadin is OD_{620}=1 to 250 ug/ml gliadin. This data can be a good reference for *ex vivo* and *in vitro* test. For *ex vivo* study by using TIM-1 model (Mitea et al., 2008), the amount of food is 70 g bread, which contains 5 g gluten, indicting 2.5 g gliadin. According to our result, 250 µg/ml gliadin was degraded in an OD=1. Therefore, OD=10,000 will be needed for the future TIM-1 experiment.

Nowadays, there are some probiotics in the market, especially there is one specific for CD, the CeliAct (CeliAct). It is composed of multivitamin including vitamin A, C, D, E, K, etc., and mineral complex; probiotic defense complex including *Lactospore* complex, *Lactobacillus acidophilus* complex, and *Bifidobacterium lactis* complex; and digestive enzyme support complex including lipase complex, Amylase complex, peptidase complex etc. All these complex have to pass the generally recognized as safe (GRAS). With the support of the future *in vitro* and *in vivo* experiments, *R. aeria* enzyme is potentially to be applied as a probiotic by degrading gluten.

In summary, full strength BHI broth and 20 to 44 hr cultivation at 37°C are considered as optimal cultivation condition to obtain a *R. aeria* cell culture with high enzyme activity. Starvation condition do not enhance enzyme expression.

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CURRICULUM VITAE

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